



**Interaction between *Mycobacterium avium*
strains and human and avian host cells**

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Abstract

Avian mycobacteriosis is a chronic infectious disease of poultry caused by different mycobacteria belonging to the *M. avium*-intracellular complex but primarily subspecies *avium*.

Data on the interaction between *M. avium* and avian cells is very limited. This study describes the invasion and intracellular survival of *M. avium* isolates from both chicken and calf sources infecting THP-1-human-like macrophages and HD11 avian macrophage-like cells. There was strain to strain variation in initial invasion between the isolates tested but this variation was independent of the host-source of isolate. Invasion of the host cells was increased in the presence of host specific serum, both calf and poultry serum enhanced *M. avium* invasion of avian cells not seen with human cells and human serum increased the bacterial invasion of human cells, but not seen with avian cells. Both mannose and scavenger receptors were the dominant receptor for uptake of bacteria in human (THP-1) and avian (HD11) cells respectively. Simultaneous blocking mannose-, scavenger- and complement receptors did not result in complete inhibition of bacterial invasion in both cell lines suggesting that the phagocytic receptors are not working independently and may cooperate to interact with diverse ligands on the bacterial surface simultaneously for optimal binding and internalization. Invasion was both actin and tubulin dependent. The inhibitory effects of combinatory blocking of receptors and cell

cytoskeleton are synergistic on the bacterial invasion of both THP-1 and HD11 cells. Post-invasion, nitric oxide and reactive oxygen species played different roles in the intracellular bacterial survival. Nitric oxide production was correlated with a reduction in intracellular survival of *M. avium*. In contrast, induction of reactive oxygen species enhanced *M. avium* survival and inhibition of reactive oxygen species using antioxidants led to a significant reduction in bacterial survival. Entry via specific receptors could have significant effect on the bacterial survival within the host cells. *M. avium* infection of HD11 can lead to disruption of tubulin and subsequent inhibition of both the phagosomal acidification and lysosome fusion so enhancing intracellular survival of the bacterial strains within the infected cells. In conclusion, the differences observed between bacterial isolates and host cells being infected suggest subtle differences in initial invasion and survival mechanisms between different isolates and hosts cells some of which are strain dependent and some are host cell dependent.

Declaration

I hereby declare the work presented in this thesis was carried by me, the contents of this thesis, in full or in parts, have not been submitted to anywhere for any award. Any sources of information have been used, they have been acknowledged.

Nawzat Issa

May 2016

Conferences (Oral and Poster presentations)

- I. Nawzat A. Issa, Mohammed Shukur, Sabine Töttemeyer, Paul Barrow, Michael A. Jones. 2014. The role of cellular scaffolding in invasion and intracellular survival of *M. avium* subspecies *avium* in host cells. In: Proceedings of the Kurdistan Students Conference 2014, Nottingham, United Kingdom.
- II. Nawzat A. Issa, Mohammed Shukur, Sabine Töttemeyer, Paul Barrow, Michael A. Jones. 2014. Roles of innate antimicrobial mechanisms in intracellular survival of *M. avium* in chicken cells. The 2014 Innate Immunity Summit. London, United Kingdom.
- III. Nawzat A. Issa, Mohammed Shukur, Sabine Töttemeyer, Paul Barrow, Michael A. Jones. 2014. Invasion and intercellular survival of *Mycobacterium avium* subspecies *avium* in chicken and human macrophages. The 3rd Prato Conference on the Pathogenesis of Bacterial Diseases of Animals 2014. PRATO, ITALY.
- IV. Nawzat A. Issa, Mohammed Shukur, Sabine Töttemeyer, Paul Barrow, Michael A. Jones. 2015. Interaction between *M. avium* and avian macrophages. Global alliance for research on avian diseases (GARAD) 2015. London. United Kingdom.

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List of Abbreviations

AHC	alkylhydroper-oxide reductase
AO	Auramine O-rhodamine stain
BCG	Bacille Calmette–Guérin
C3bBb	C3 convertase
CD	cytochalasin D
CFU	colony forming units
CHS	Chicken serum
CR3	complement receptors 3
Cu-ZnSOD	superoxide dismutase
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DPI	diphenyleneiodonium chloride
EDTA	disodium ethylene diamine tetra acetate
EEA1	early endosomes markers
EGTA	ethyleneglycol-bis (beta-amino-ethyl ether) N, N'-tetraacetic acid
FAM	fluorescent acid-fast microscopy
FCS	foetal calf serum
HD11	Chicken macrophages-like cell line
HIS	heat inactivated sera
HS	Human serum
iNOS	nitric oxide synthase
KatG	catalase-peroxidase
LAM	lipoarabinomannan
LAMP-1	lysosomes markers
LDH	lactate dehydrogenase

List of Abbreviations

<i>L</i> -NMMA	<i>N</i> ^G -Monomethyl- <i>L</i> -arginine, monoacetate
LPS	lipopolysaccharide
MAC	<i>Mycobacterium avium-intracellulae</i> complex
ManLAM	mannose-capped lipoarabinomannan
MASP	mannose binding lectin-associated serine protease
MBL	mannose-binding lectin
MHC I	major histocompatibility complex class one
MHC II	major histocompatibility complex class two
MnSOD	manganese-dependent superoxide dismutase
MOI	multiplicity of infection
MR	mannose receptors
NAC	N-acetyl-L-cysteine
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NBT	nitroblue tetrazolium
NED	(0.1% N-1-naphthylethylenediamine dihydrochloride in water)
NHS-CF	5(6)-Carboxyfluorescein <i>N</i> -hydroxysuccinimide ester
NHS-Rho	NHS-rhodamine
NO	nitric oxide
Noco	Nocodazole
O ₂ ⁻	superoxide anion
OADC	oleic acid albumin dextrose catalase
ONOO ⁻	Peroxynitrite
OSO ₄	osmium tetroxide
PBS	phosphate buffer solution
PDIMs	phthiocerol dimycocerosates
PGLs	phenolic glycolipids

PI	post-infection
PILAM	phosphatidylinositol caps on the LAM
PIMs	phosphatidylinositol mannosides
PMA	phorbol-12-myristate-13-acetate
RAB7	late endosomes markers
rchIFN- γ	recombinant chicken gamma interferon
RNI	reactive nitrogen intermediates
RNS	reactive nitrogen species
ROI	reactive oxygen intermediates
ROS	reactive oxygen species
SLs	sulpholipids
SOD	superoxide dismutase
SR A	Scavenger receptors class A
SRs	Scavenger receptors
TACO proteins	tryptophan-aspartate-containing coat protein
TDM	trehalose 6, 6' – dimycolate
TEM	Transmission Electron Microscopy
THP-1	human macrophages-like cell line
TNF- α	tumour necrotizing factor

1.0 Introduction

Avian mycobacteriosis is a chronic infectious disease of birds mainly caused by *Mycobacterium avium*. The disease most frequently occurs due to *M. avium* serotypes one, two, three, and six. In addition, other species have been reported to cause avian mycobacteriosis, such as *M. tuberculosis*, *M. bovis*, and *M. fortuitum*, but their occurrence are very rare (Grange et al., 1990, Dvorska et al., 2007). In infected birds, clinical signs of the disease vary depending on the organs affected, but are generally characterized by a chronic and progressive wasting, commonly associated with enteritis. Economically, the disease is classified as an important infectious disease because of both direct and indirect losses (Mutalib and Riddell, 1988, Quaranta et al., 1996).

1.1 Mycobacterial taxonomy

In 1896, Lehmann and Neumann classified the genus of *Mycobacterium* for the first time according to their morphology and acid-alcohol fastness (Lehmann and Neumann, 1901). Three major criteria should be fulfilled for bacteria to be classified within the genus. These are acid-alcohol fast, according to the acid fast stains, presence of mycolic acids in the bacterial envelope and a bacterial genome GC content of 61-71% (Lévy-Frébault and Portaels, 1992). The genus *Mycobacterium* locates to the family *Mycobacteriaceae*, which belongs to the phylum *Actinobacteria* (Ventura et al., 2007).

Mycobacteria are non-motile, non-spore-forming, aerobic bacteria, rod shape, and 1-10 μm long (Draper, 1998).

1.2 Mycobacterial cell wall

A unique and complex cell wall is one of the most important properties of mycobacteria (Figure. 1.1 and I.2). The wall is highly lipophilic with diversity in lipid structures and exhibits a limited permeability to water. The complex wall is believed to play roles in protection against hostile environments within the host cells and resisting the influx of antibiotics (Draper, 1998).

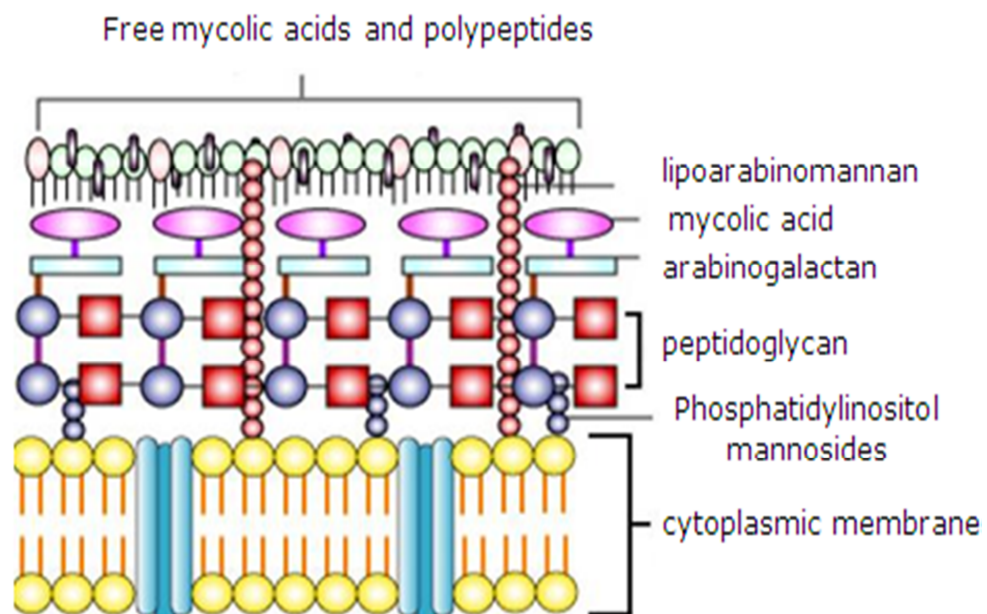


Figure 1.1: Overview of the structure of the cell wall of *M. tuberculosis* (Nature Reviews Microbiology 5, 883-891)

The bacterial cell wall is divided into three major components; the plasma membrane, the mycolic acid-arabinogalactan-peptidoglycan complex and the surface capsule. Mycolic acids are long, branched

2-alkyl-3-hydroxy fatty acids that may be as many as 90 carbon atoms in length (Draper, 1998, Brennan, 2003). The cell envelope also contains a variety of loosely attached polar lipids and lipoglycans that are unique to mycobacteria. While the types of lipid present vary from one mycobacterial species to another, some commonly occurring ones include phosphatidylinositol mannosides (PIMs) and lipoarabinomannans (LAMs) (Draper and Daffé, 2005). PIM is the precursor of lipomannan, which in turn is the precursor of LAM (Draper, 1998). PIMs have been shown to have a role in natural killer cell recruitment as well as adhesion to non-phagocytic host cells (Kremer and Besra, 2005). The type of LAM present in the species correlates with its pathogenic ability. For example, mannose caps, present on LAM (ManLAM) in *M. tuberculosis*, *M. leprae* and *M. avium* (Figure. 1.2) make ManLAM a much stronger immunosuppressor as compared to PILAM of *M. smegmatis*, which contains phosphatidylinositol caps on the LAM (Khoo et al., 1995) and AraLAM is free from mannose caps in *M. chetanae* (Brennan, 2003, Kremer and Besra, 2005). A LAM without the arabinan part, termed lipomannan, is also present in the cell wall (Besra et al., 1997).

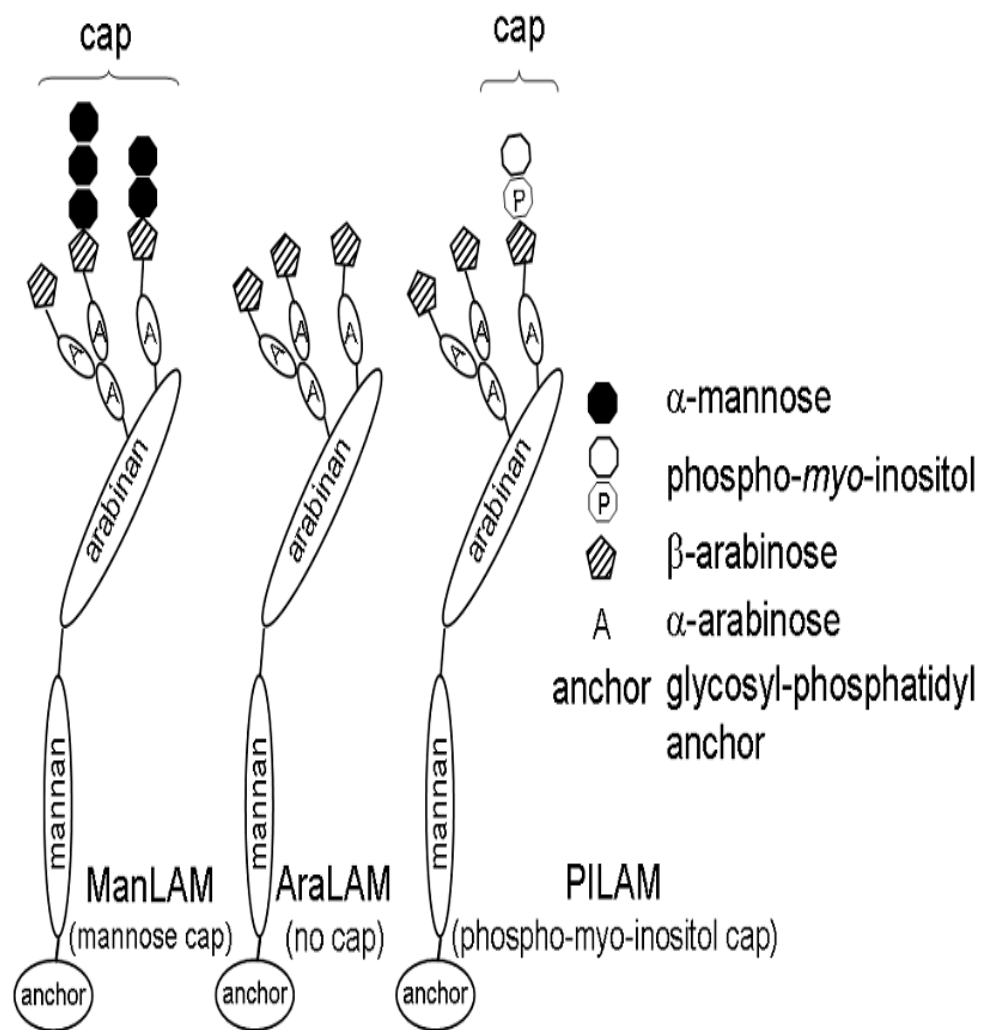


Figure 1.2: Types of lipoarabinomannans: ManLam, AraLam and PILAM from *M. tuberculosis*, *M. chetanae*, and, *M. smegmatis*, respectively. Adapted from references (Guérardel et al., 2002, Dao et al., 2004).

M. tuberculosis has special classes of mycolic acids that including trehalose-6,6'-dimycolate (TDM) that are commonly referred as 'cord factor', sulpholipids (SLs), phthiocerol dimycocerosates (PDIMs), phenolic glycolipids (PGLs) (Brennan, 2003, Kremer and Besra, 2005, Torkko et al., 2003) and glycopeptidolipids (Horgen et al., 2000). A range of biological effects have been attributed to the different groups of mycolic acids (Table 1).

Table 1.1: The biological effects associated with mycobacterial mycolic acids.

Lipid group	Biological effects	References
Trehalose dimycolate	Participates in caseation and granuloma formation. Triggers the release of IL-1 β TNF, IL-6, and IL-12 in murine macrophages	(Guidry et al., 2007), (Hunter et al., 2006)
Sulpholipids	Suggested to play a role in phagosomal maturation; however, it is still unclear. Stimulates the release of oxygen radical by neutrophils	(Brennan, 2003)
Phthiocerol dimycocerosates	The major bacterial waxes acting as permeability barrier	(Simeone et al., 2007)
Phenolic glycolipids (PGLs)	TNF induction by human monocytes Induction of antibodies in leprosy patients Triggers release of oxygen radical production by neutrophils	(Cho et al., 2001)
Glycopeptidolipids	Reduces IFN- γ production by human PBMC	(Horgen et al., 2000)

1.3 Avian mycobacteriosis

Avian mycobacteriosis is a progressive wasting disease, it can affect a wide range of bird species and may infect a number of mammalian species. Avian mycobacteriosis can be seen in different forms; such forms include enteritis (Hoefer et al., 1996, Mutalib and Riddell, 1988), respiratory (Hoop et al., 1993, Portaels et al., 1996), skin lesions (Bowes, 1993, Britt Jr et al., 1980) and nervous (Lumeij et al., 1980, Lairmore et al., 1985). Various factors like gender, age, stress and immune state have been considered to play roles in severity of the disease (Hoenerhoff et al., 2004).

1.3.1. Distribution and sources of infection

Avian mycobacteriosis is not restricted geographically although it is reportedly uncommon in certain countries such as Japan (Morita et al., 1994) and most frequently reported in the North Temperate Zone (Dhama et al., 2011). Surface water including ponds, lakes, rivers and streams are considered to be the most common source for *M. avium* (Grange et al., 1990). The *M. avium* complex has been isolated from soil enriched with organic matter or animal manure (Grange et al., 1990). Mycobacteria can persist in soil for years; thus contaminated soil remains an important source for following generations of poultry (Mutalib and Riddell, 1988). Ingestion of the organism through contaminated food and water is the most frequent route for mycobacterial transmission (Fulton and McCallion, 1987, Dvorska et al., 2007). The respiratory tract is also assumed as an important route of infection. Skin contacts with infected materials and vertical transmission have also been reported to transmit the organism (Fulton and McCallion, 1987). Other species such as rats and other rodents can act as vectors to transfer the bacteria (Fulton and McCallion, 1987, Dvorska et al., 2007).

1.3.2 Species susceptibility

Birds can be classified into four groups according to their susceptibility to avian mycobacteriosis: The first group includes very sensitive species such as domestic fowl, sparrows, pheasants, and partridges, while the second group is classified as less sensitive

species including Guinea fowl and domestic turkeys. Domestic geese and duck are included in the third, moderately resistant group, while a highly resistant species is the domestic pigeon (Hejlícek and Tremł, 1995, Dvorska et al., 2007).

1.3.3 Sex and age

In birds, both males and females appear to be equally sensitive to the disease (Keymer et al., 1982). However, it has been reported that turkey males are less susceptible to infection than females (Hejlícek and Tremł, 1995). Moreover, it has been shown in two reports, involving psittacine and passerine birds, that the disease was slightly less common in males than in females (Hoop et al., 1993). Young birds are more susceptible to the disease than the adults (Cromie et al., 1991, Mutalib and Riddell, 1988). In the UK, it has been found that the mortality rate following infection in adult swans was 33% comparing to 4% in young swans (Brown et al., 1992). This finding reflects the chronic nature of the disease, as under natural conditions the disease begins in very young birds and requires long periods before inducing significant effects on animals with good body condition (Feldman, 1938, Soler et al., 2009, Dhama et al., 2011).

1.3.4 Clinical signs

Clinical signs of the disease vary depending on the organs involved. Clinically the disease is characterized by chronic and progressive

wasting and diarrhoea can be common (Saggese et al., 2010, Dahlhausen et al., 2012). Respiratory signs may develop in some birds and occasionally sudden death occurs (Hoop et al., 1993). Some birds may develop granulomatous ocular lesions (Stanz et al., 1995, Pollock, 2006). The liver may be easily palpable due to emaciation of animal and abdominal distension and ascites occur as a result of hepatomegaly in infected waterfowl (Tell et al., 2001, Ledwoń et al., 2014). Nervous signs, a consequence of central nervous system and vertebral involvement, are occasionally reported (Zeller et al., 2003). Lameness, as a complication of bone or joint involvement, can also develop (Pond and Rush, 1981, Stroud et al., 1986).

1.3.5 Treatment of mycobacteriosis in birds

M. avium is highly resistant to antimicrobial drugs. The treatment of the disease is very difficult, due to the high cost of the drugs and prolonged time of the therapy (Tell et al., 2001, Buur and Saggese, 2012). A highly lipophilic mycobacterial cell wall acts as a restrictive barrier and protects *M. avium* from hydrophilic antibiotics such as aminoglycosides, fluoroquinolones, and macrolides. In addition, *M. avium* is slow growing and is intracellular necessitating extended periods of therapy (VanDerHeyden, 1997). Because of inherent resistance to antimicrobial therapy and economic considerations the treatment of the disease is not considered in the poultry industry.

1.4 Immune response against mycobacterial infection

Published literature on the immune response against *M. avium* is very limited; most is focused on *M. tuberculosis* and *M. bovis*. Therefore, this study has used *M. tuberculosis* as a model with which to explain the immune response in mycobacterial infection. The innate immune response plays a significant role in the defence mechanism against different pathogenic organisms including mycobacteria. An effective innate immune response against *M. tuberculosis* infection can prevent development of the disease or may be able to completely stop the infection in some patients (Ewer et al., 2006, Dheda et al., 2010). *M. tuberculosis* hide themselves from some components of the innate immune response within macrophages. The bacteria exist and replicate within the host macrophages following inhibition of phagosome maturation or inhibition of acidification of the phagosomes-lysosome, and avoiding oxidative stress by expressing lipid and protein (Meena and Rajni, 2010).

Macrophages are cells of the innate immune system and professional phagocytic cells. These cells are thought to play a crucial role in mycobacterial infection particularly due to *M. tuberculosis*. The cells can be activated via different stimuli including bacterial LPS, IFN- γ and tumour necrotizing factor TNF (Lewis et al.,

1990). Activated cells are characterized by their ability to eliminate invading pathogens. However, some intracellular organisms such as *M. tuberculosis* have developed different ways to interfere with the cell responses in order to survive (Benoit et al., 2008, Flannagan et al., 2009). Activated macrophages kill intracellular pathogens via production of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) (Chan et al., 1992, Denis, 1991, Bartos et al., 2004). Infected macrophages release different cytokines in response to the bacterial infection and present mycobacterial antigens to cytotoxic T cells via major histocompatibility complex class one (MHC I) and to CD4+ T helper cells through MHC II (Dheda et al., 2010). The released cytokines from the infected macrophages include IL-7, IL-12, IL-15, IL-23 and TNF- α which stimulate other cells to move toward the infection site (Dheda et al., 2010).

1.5 Invasion mechanisms and the interactions between receptors and cytoskeleton.

Pathogenic mycobacteria have developed different strategies to tolerate host innate antimicrobial activities and to replicate within the intracellular organelles of the infected cells. The phagocytosis of pathogens is a complicated process can be divided into three successive steps: (1) interactions between bacterial ligands and host cell receptors (2) formation of the phagocytic fold membrane through actin polymerization and extension of the cell membrane

(3) internalization of the microorganism which also depends on the actin polymerization (Cossart and Sansonetti, 2004).

1.5.1 Role of macrophages receptors in *M. avium* invasion

Attachment and internalisation of mycobacteria into macrophages is a critical early step in the establishment of the disease. Studies suggest that the phagocytosis of mycobacteria can follow serum-dependent or independent routes. Serum-dependent invasion of phagocytic cells occurs more rapidly (Bermudez et al., 1991, Bohlson et al., 2001). *M. tuberculosis* interacts with various receptors on the surface of mammalian macrophages, such receptors including complement receptors (Bermudez et al., 1991, Hirsch et al., 1994, Roecklein et al., 1992, Schlesinger et al., 1990, Schlesinger, 1993), mannose receptors (Bermudez et al., 1991, Schlesinger et al., 1990), scavenger receptors (Dunne et al., 1994, Krieger et al., 1993), surfactant protein receptors in the lung (Gaynor et al., 1995) and FC receptors (Laal et al., 1997, Armstrong and Hart, 1975). The relative contribution of these receptors to *M. tuberculosis* survival within the infected macrophages is not fully understood (Ernst, 1998).

1.5.1.1 Role of serum in mycobacterial invasion

Phagocytosis of mycobacteria by macrophages is assumed to be an important step in the life cycle of the organism. Studies suggest that

invasion of mycobacteria by macrophages can be increased in the presence of serum. 5% to 20% serum is crucial to obtain an optimal interaction between cultured bovine blood monocytes and monocyte-derived macrophages and *M. paratuberculosis* (Zurbrick and Czuprynski, 1987). The invasion of human macrophages by pathogenic mycobacteria such as *M. tuberculosis*, *M. leprae*, and *M. avium* is dependent on the presence of the human serum (Schorey et al., 1997). There are suggestions from murine cell invasion studies that *M. avium* invasion is increased by the presence of serum particularly during the first two hours post-infection (Bermudez et al., 1991). It has been found when mycobacteria are sufficiently coated with the complement proteins they are rapidly phagocytized via complement receptors (Hirsch et al., 1994, Schlesinger, 1993, Schlesinger et al., 1990, Roecklein et al., 1992).

The complement system is regarded as a main component of the innate immune response in vertebrates; the system composes of about 30 soluble proteins (Dunkelberger and Song, 2010). Complement is a heat-sensitive protein which enhances coating of pathogens with antibodies; the complement proteins enhance antibodies to kill some pathogens, this activity is known to 'complement' the antibacterial activity of antibodies. The complement system can be activated in three different ways (Figure 1.3): the classical pathway (Rus et al., 2005), the lectin pathway (Bartłomiejczyk et al., 2014) and the alternative pathway (Thurman

and Holers, 2006, Janeway et al., 2001). Each pathway has been seen to be stimulated by specific stimuli (Janeway et al., 2001). The classical pathway mainly depends on the interaction between antigen-antibody complexes with C1q (Rus et al., 2005). The interaction causes activation of the C1s and C1r leading to formation of C1s esterase, which is needed to cleave C4 and C2 and to generate a small amount of C4bC2a, a C3 convertase. The C3 convertase is required to cleave C3 producing C3b and C3a (Rus et al., 2005). C3b and its products iC3b mediate the binding between the pathogens and complement receptors, mainly CR3 (Ross et al., 1999). Activation of the complement system by the lectin pathway is initiated by interaction between mannose-binding lectin (MBL) or ficolins with a series of carbohydrates on the bacterial surfaces (Bartłomiejczyk et al., 2014). MBL and ficolins attach and activate mannose-binding lectin-associated serine protease (MASP) 1, 2, and 3. The activated MASP cleaves C4 and C2 leading to production of C3 convertase (Reid and Turner, 1994, Dahl et al., 2001). Activation of the system by the alternative pathway can occur in the absence of antibodies. Under normal conditions the complement component C3 undergoes a spontaneous cleavage leading to form C3a and C3b fragments within the serum and C3b can bind to different surface (Rus et al., 2005). The deposited C3b is bound by factor B which is cleaved by factor D, leading to produce Bb. The Bb produced binds to C3b and results in the formation of C3 convertase, C3bBb (Thurman and Holers, 2006, Rus et al., 2005).

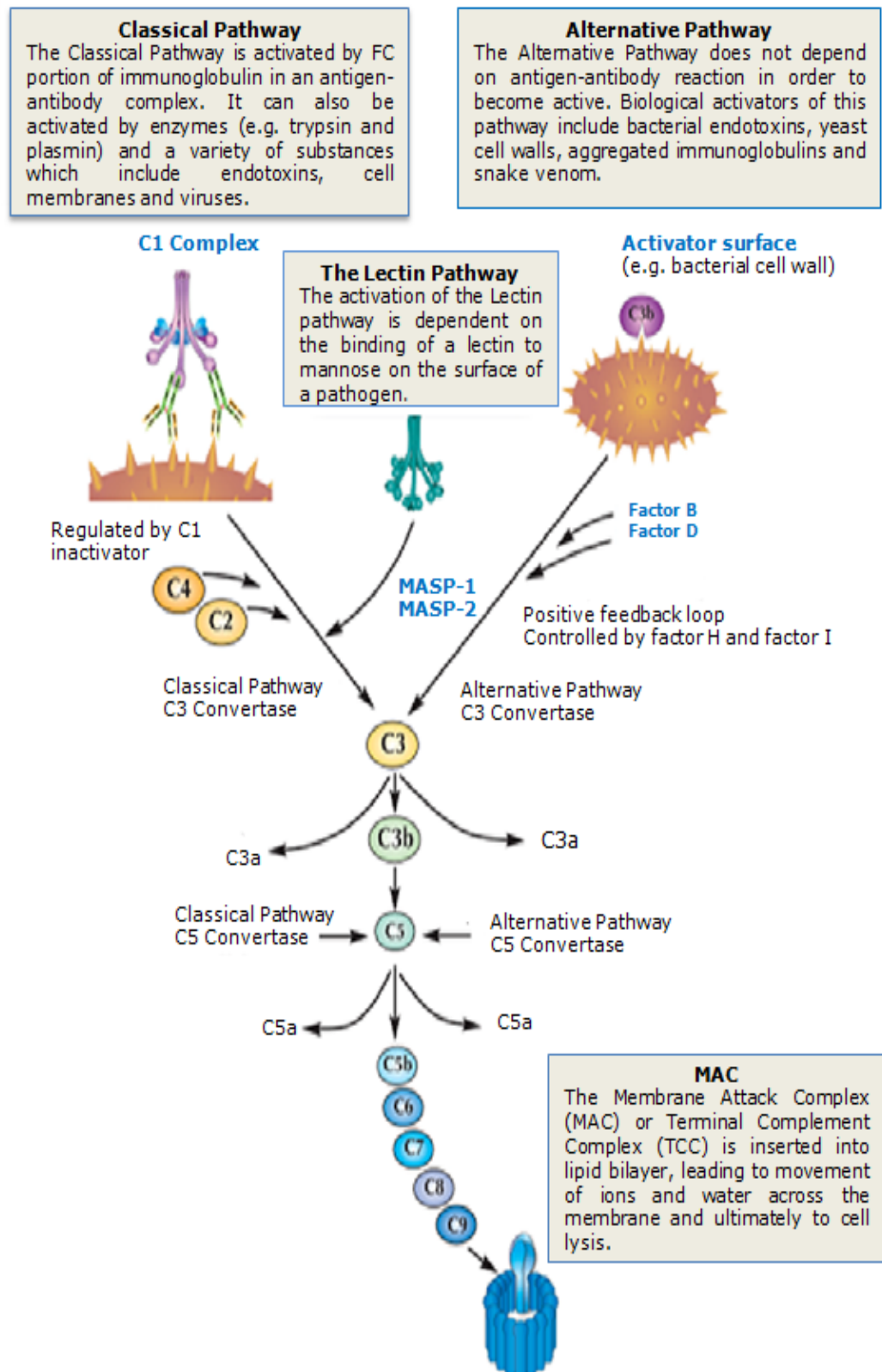


Figure 1.3: Activation of the complement system via classical, alternative and lectin pathways.
(<http://www.thebindingsite.com/complement>).

Mycobacteria activate the complement system in different ways. Various species of pathogenic mycobacteria such as: *M. tuberculosis*, *M. bovis*, *M. kansasii* and *M. goodii* have been demonstrated to activate the complement system via the lectin pathway which enhanced the bacterial ingestion by human phagocytes (Bartłomiejczyk et al., 2014). It has also been reported that *M. tuberculosis* activates the complement system by both antibody-dependent classical pathway and alternative pathway leading to increase bacterial phagocytosis by human alveolar macrophages via complement receptors (Ferguson et al., 2004). Manivannan et al. (2012) reported that antibodies IgG raised against *M. tuberculosis* played significant roles in activation of the complement system and phagocytosis of the bacteria by human macrophages. Besides, *M. tuberculosis*, *M. leprae* and *M. avium* have been shown to form C3 convertase by binding to C2a even in the absence of C4b to produce C3b which essential for bacterial opsonisation (Schorey et al., 1997). Consistent with this mechanism, heat-inactivated of complement components such as C2a and C3b lead to a decreased invasion of human macrophages by *M. tuberculosis* (Schlesinger et al., 1990). Complement receptors, namely CR3, binds to C3bi, a cleavage product of complement component C3. C3bi plays an important role in mycobacteria opsonisation and subsequent invasion of macrophages via complement receptors (Sarrias et al., 2001).

1.5.1.2 Complement receptors

Laboratory studies reported that mycobacteria use different receptors to invade phagocytes. Complement receptors play important roles in phagocytosis of pathogenic mycobacteria (Manivannan et al., 2012, Hossain and Norazmi, 2013). Different complement receptors are found on various phagocytic cells, such receptors including CR1 (CD35), CR2 (CD21), CR3 (CD11b/CD18) and CR4 (CD11c/CD18) (Eggleton et al., 2000, Bajic et al., 2013). Coating of mycobacteria with C3b promotes the bacterial invasion by human phagocytic cells through complement receptors (Bermudez et al., 1991, Hirsch et al., 1994, Roecklein et al., 1992, Ferguson et al., 2004, Schlesinger et al., 1990, Schlesinger, 1993, Cywes et al., 1997, Stokes et al., 1993). Among the complement receptors, CR3 is the most important receptor for bacterial invasion (Hossain and Norazmi, 2013). CR3, also known as Mac-1, $\alpha\text{M}\beta\text{2}$ and Mo-1, is a member of β2 integrin family which is composed of two chains, a CD11b alpha chain of 170 kDa and a CD18 beta chain of a 95 kDa, the two chains being linked non-covalently (Ross and Medof, 1985). CR3 plays an important role in *M. tuberculosis* invasion of human macrophages (Caron and Hall, 1998). Invading macrophages via complement receptors is regarded as a virulence mechanism of the bacteria (Ernst, 1998). It has been reported that the invasion of human macrophages and monocytes by *M. tuberculosis* was reduced 80% by blocking CR3 (Schlesinger et al., 1990). In contrast to the

other receptors, namely mannose receptors, CR3 can interact with virulent and avirulent strains of *M. tuberculosis* (Schlesinger et al., 1990).

The role of CR3 in the intracellular survival of *M. tuberculosis* in mammalian macrophages is controversial, Zimmerli et al. (1996) found no effect of CR3 deficiency on the intracellular survival of *M. tuberculosis* in human macrophages. Also no effect of *M. tuberculosis* infection on the survival of mice deficient in CD11b was observed (Melo et al., 2000). In contrast, it has been reported that *M. tuberculosis* invasion via CR3 interfered with ROS production by mammalian infected macrophages, hindered the phagolysosome maturation process and with no inflammatory responses (Sturgill-Koszycki et al., 1997). Interaction of human macrophages and Zymosan via complement receptor CR3 induced reactive oxygen species production, whereas the interaction between viable or heat-killed *M. kansasii* did not (Le Cabec et al., 2000). Interaction of human macrophages and *M. tuberculosis* via complement receptors provides the bacteria safe entry and allowing them to avoid the toxic consequences of ROS (Schlesinger et al., 1990). Due to this vital role of CR3 in invasion of mammalian macrophages and the uncertain role in the kinetics of the mycobacterial survival within human macrophages sections this study focused on studying the role of CR3 in invasion and intracellular survival within chicken macrophages and compared with human macrophages.

1.5.1.3 Mannose receptors

The mannose receptor (MR) was described for the first time by Stahl and co-workers as a 175kDa type I transmembrane protein involved in the clearance of extracellular hydrolases (Stahl et al., 1976). Structurally, mannose receptors contain eight carbohydrate recognition domains, domains 4, 5 and 7 are recorded to be the most important to bind and internalize ligands of oligosaccharides of mannose, fucose or *N*-acetylglucosamine (Taylor et al., 1992). Different pathogens including mycobacteria have been reported as physiological ligands for mannose receptor (Lane et al., 1998). The mannose receptors interact with mycobacterial glycolipids such as mannose-capped lipoarabinomannan (ManLAM) and phosphatidylinositol mannosides (PIMs) (Driessen et al., 2009). Although, *M. tuberculosis* utilizes these receptors to invade human cells (Kang et al., 2005), human mannose receptor expressed in Chinese hamster ovary (CHO) cells were unable to internalize *M. kansasii* or mannosylated latex beads compared to phagocytosis of mannosylated glycoprotein. It has been suggested that mannose receptor is a binding receptor not a professional phagocytic receptor as the receptor was unable to internalize particle in non-phagocytic cells (Kang et al., 2005). The receptor needs to cooperate with the other receptors to induce phagocytosis in phagocytic cells such as macrophages (Le Cabec et al., 2005). Differences have been reported between virulent and avirulent strains of *M. tuberculosis*

with regards to their ability to exploit the mannose receptor and gain entry into human macrophages; attenuated strains of *M. tuberculosis* such as H37Ra were unable to interact with mannose receptors and adhere to macrophages compared with the virulent strains, Erdman and H37Rv (Schlesinger, 1993). The difference between the two strains is due to the variation within the bacterial cell wall structure, compared to the attenuated strain of *M. tuberculosis*, lipoarabinomannan of virulent strain is associated with mannose oligosaccharide "caps" at the terminal portions of the molecule that mediated the bacteria binding to mannose receptors (Chatterjee et al., 1992)

Although, the precise role of mannose receptors in bacterial survival is unknown, pathogenic mycobacteria utilize mannose receptors to enter into the host cells and escape from the immune response (Schlesinger, 1993). The BCG bacillus was reported to down regulate expression of mannose receptor in murine macrophages (Ezekowitz et al., 1981). Furthermore, it has been found that the entry of *M. avium* through mannose receptors to human macrophages enhances bacterial survival, since mannose receptors-dependent invasion interfered with phagosome-lysosome maturation (Shimada et al., 2006). In contrast to FcRγ and CR3-phagosomes, mannose receptor phagosomes associate with F-actin (Kerrigan and Brown, 2009). F-actin polymerization is required for mannose receptor -mediated phagocytosis (Kerrigan and Brown, 2009, Zhang

et al., 2005). Expression of mannose receptors relies on F-actin. Depolarization of human alveolar macrophage actin reduces mannose receptor expression and function (Zhang et al., 2005). However, the importance of mannose receptor in the phagocytosis of *M. avium* by chicken macrophages is unknown; the interaction between the receptor and cell cytoskeleton needs to be examined, but it could be predicted that the receptors will play an important role in the absence of serum. Studying the interactions between the cell cytoskeleton and the mannose receptor, the receptor-mediated phagocytosis and its effects on the bacterial survival will be valuable in assessing whether mannose receptors play a significant role in *M. avium* invasion and survival in chicken cells.

1.5.1.4 Scavenger receptors

Scavenger receptors (SRs) are membrane glycoproteins with the ability to interact with a chemically modified low-density lipoprotein (Peiser et al., 2002). The receptors are classified into three classes: class A, B, and C (Peiser et al., 2002). Scavenger receptors class A (SR A) are homotrimeric glycoproteins made up of collagen and can be divided into three distinct kinds: Type I, II and III, the classification based on the number of amino acids of the cysteine-rich domain at the C terminus of the protein (Gough and Gordon, 2000). Scavenger receptors class A are widely expressed in macrophages in different tissues and act as pattern recognition molecules (Peiser et al., 2002). In addition, Scavenger receptors

class A is made up of collagenous structure (MARCO) found on alveolar macrophages and dendritic cells of human and murine macrophages (Granucci et al., 2003, Areschoug and Gordon, 2009). Scavenger receptor class B or CD36 is a glycoprotein found in different cells including macrophages (Murphy et al., 2005). Scavenger receptors class A bind to *M. tuberculosis* through its cell surface sulfolipid component (Ernst, 1998). Peiser et al. (2000) found that scavenger receptors class A bind and ingest bacteria directly even in the absence of the opsonizing agents. By using competitive inhibitors, it has been found that scavenger receptor class A is significant for pathogenic strains of *M. tuberculosis* Erdman strains to invade human monocyte-derived macrophages (Zimmerli et al., 1996). It has also been shown that the mycobacterial burden of *M. bovis* BCG in liver and spleen in Cd36^{-/-} (SR B^{-/-}) mice reduced to 83% relative to the wild-type Cd36^{+/+} (Hawkes et al., 2010). *M. tuberculosis* can alternatively use scavenger receptors to invade host cells, when bacterial invasion via complement receptors was inhibited (Zimmerli et al., 1996) .

Scavenger receptors have been reported to play a role in controlling of *M. bovis* BCG infection since the deficiency of the receptor limited bacterial survival within murine cells (Hawkes et al., 2010). It has been suggested that the bacteria use the receptor to evade immune responses of the host (Hawkes et al., 2010). It has been found that the resistance of mice to acute or chronic *M. tuberculosis* infection

was impaired by a deficiency of a scavenger receptors class A and CD36 (Vasseur et al., 2010). It has been reported that dextran sulfate, a general ligand of scavenger receptors, suppresses nitric oxide production by LPS-treated murine macrophages (Józefowski et al., 2011). Meena and Rajni (2010) stated that the pathogenic strategy of using a selective receptor for invasion and intracellular survival of *M tuberculosis* is not fully understood. However, it is possible that the macrophage responses via secretion of different cytokines are affected by the distinct entry routes or downstream activation of signals in infected cells resulting in differential survival of the bacteria.

The interaction between scavenger receptors and cell cytoskeleton is not yet known and it is unclear to what extent the receptor can rely on actin and tubulin for internalization of bacteria, also it is unknown whether scavenger receptor is binding receptor or actual phagocytic receptor. In the context of mycobacterial infection nothing, as yet, has been reported on the interaction between scavenger receptors and cell cytoskeleton and their effect on the bacterial invasion except Ernst (1998) stated that the interactions between scavenger receptor and mammalian cells cytoskeleton are currently undefined.

1.5.1.5 FC receptors and surfactant protein A receptors

Studies have been done using a murine model of FC receptor deficiency to study the role of the receptor in intracellular

pathogens, including *Chlamydia trachomatis*, *Leishmania* species and *Salmonella enterica* (Moore et al., 2002, Woelbing et al., 2006, Uppington et al., 2006). In the context of mycobacterial infection it is not clear how relevant this would be as humoral immunity appears to have little or no role in host-defence against the mycobacterial infection (Maglione et al., 2008). However, some infected individuals have circulating antibodies to *M. tuberculosis*, and it has been reported that the intracellular trafficking of the pathogenic strain of *M. tuberculosis* (H37Rv) coated with immunoglobulin is different from that of non-opsonized bacteria in infected murine macrophages (Laal et al., 1997). The mycobacteria coated with immunoglobulin G (IgG) are ingested by macrophages in vesicles which bind with ferritin-loaded lysosomes (Fig 1.4), whereas, non-opsonized mycobacteria are found in phagosomes that do not acquire ferritin from labelled lysosomes (Ernst, 1998). It has been suggested that the ingestion of *M. tuberculosis* through Fc receptors may lead to a distinct intracellular trafficking pathway for the bacteria but this route does not affect intracellular growth of the bacteria (Ernst, 1998).

Besides the above mentioned receptors, other cell surface proteins have also been reported to play a role in the interactions between mycobacteria and host cells; there is evidence that *M. tuberculosis* attaches and invades alveolar macrophages via lung surfactant protein (Gaynor et al., 1995, Downing et al., 1995). CD14 has been found to interact with the lipoarabinomannan and play a role in *M.*

tuberculosis invasion into both human and porcine macrophages (Shams et al., 2003, Khanna et al., 1996). CD43, a transmembrane glycoprotein (Leukosialin or sialophorin), has been reported to be required by *M. tuberculosis* strain Erdman for optimal binding to murine macrophages using murine CD43 knockout macrophages (Randhawa, 2007).

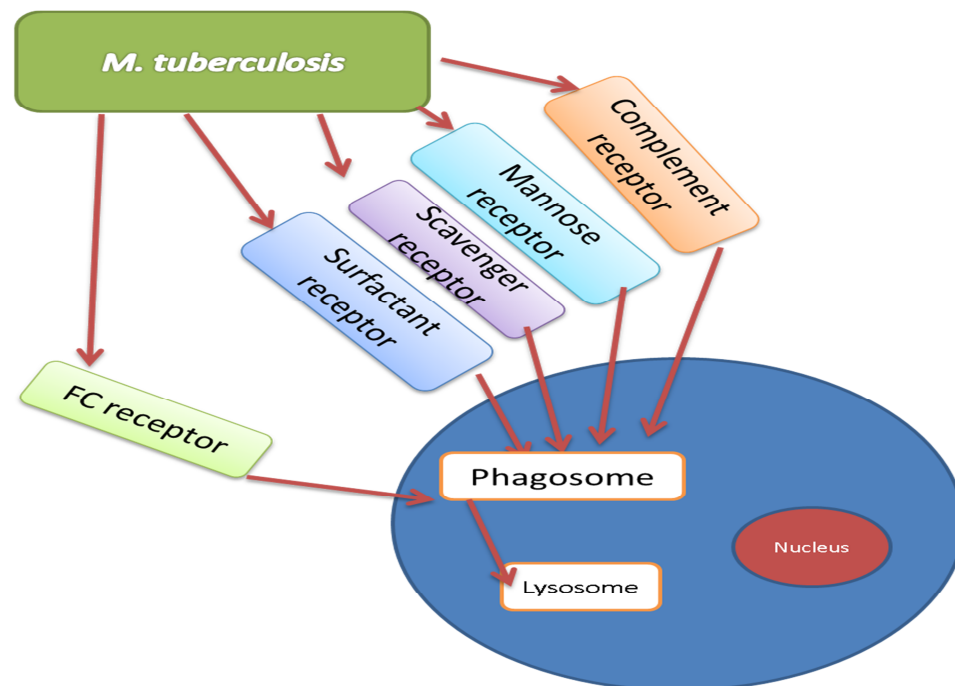


Figure 1.4: Interaction between *M. tuberculosis* and receptors on macrophages. Invasion via Complement, Mannose, Scavenger and Surfactant receptors localize mycobacteria within phagosomes. FC receptors direct the organism to lysosomes.

1.5.2 Role of the cell cytoskeleton in invasion and intracellular survival of mycobacteria within the host cells

Phagocytosis is a cytoskeleton-dependent mechanism (Greenberg and Silverstein, 1993, Kelleher and Titus, 1998). The cytoskeleton is

a series of intracellular proteins consisting of three major components: microfilaments which are made up of the protein actin, microtubules consisting of the protein tubulin (Wickstead and Gull, 2011) and intermediate filaments composed of different building block proteins only have been seen so far in animal cells (Herrmann et al., 2007). The cell cytoskeleton has different functions ranging from cell movement, adhering to surfaces, phagocytosis and trafficking of the intracellular organelles (Wickstead and Gull, 2011). Garcia-Perez et al. (2011) demonstrated that infection *M. tuberculosis* triggers actin rearrangement and membrane ruffling of mouse lung fibroblasts. *M. tuberculosis* internalization in human fibroblasts is actin and tubulin independent, but internalization does not require cell host cytoskeleton reorganization (Ferrer et al., 2009). Ferrari et al. (1999) found that actin is associated with phagosomes containing viable and dead bacilli whereas, in contrast to actin, tubulin remains associated exclusively with phagosomes containing killed bacteria suggesting that the live bacteria induced dissociation of tubulin in infected cells and interfered with phagosome-lysosomes fusion.

The amount of nitric oxide (NO) produced in response to *M. tuberculosis* infection is related with the bacterial capacity to rearrange the actin cytoskeleton (Garcia-Perez et al., 2011). *M. avium* infection of murine macrophages causes modification of actin cytoskeleton in a similar manner to that occurred after treatment of

the cells with the actin-disrupting agent, cytochalasin D (Guerin and de Chastellier, 2000). It has also been suggested that the interactions between the actin cytoskeleton and iNOS of the murine macrophage-like cell line RAW 264.7 are vital for iNOS activities. As iNOS is normally anchored to the cytoskeleton such that cytochalasin B, an actin disrupter, has been found to interfere with nitric oxide production by murine macrophages as a result of disruption of cytoskeletal integrity (Daniliuc et al., 2003). Various species of mycobacteria exploit the host cytoskeleton for transmission between neighbouring cells during infection (Stamm et al., 2003). *M. marinum* (Stamm et al., 2003) and *M. tuberculosis* (Simeone et al., 2012) polymerizes the host cytoskeleton following escape from the phagosomes to the cytosol.

The link between specific human macrophage receptors and the cell cytoskeleton has been investigated. Complement receptors in human monocyte-derived macrophages greatly depend on the cell cytoskeleton for optimal phagocytic function; cells that were treated with actin and tubulin depolarizing agents were unable to phagocytose iC3b (EC3bi) opsonized sheep erythrocytes by CR3 (Newman et al., 1991). It has also been reported in human macrophages that the mannose receptor-mediated phagocytosis of microorganisms ends by mannose, fucose, *N*-acetylglucosamine, or glucose and is an actin-dependent process (Defife et al., 1999). Actin-depolarizing agents inhibit macrophages from binding to

surfaces via mannose receptors. There is very little information on the effect of the interactions between cell surface receptors and the cell cytoskeleton on the mycobacterial invasion of the host cells, with the exception of Ernst (1998) who showed that the interaction between scavenger receptors and the cytoskeleton to internalize mycobacteria is unknown. In the context of *M. avium* infection of chicken cells, little is known on the roles of actin and tubulin in the invasion and intracellular survival of the bacteria and the interaction between the cell cytoskeleton and the surfaces receptors.

1.6 Role of reactive oxygen species (ROS) and nitric oxide (NO) in intracellular survival of *M. avium*

Phagocytic cells adopt different intracellular killing mechanisms against mycobacteria. The ingested mycobacteria are exposed to an array of inhibitory chemicals such as; reactive oxygen species (ROS) and nitrogen radicals (Yuan et al., 1995, Chan et al., 1992, Roy et al., 2004, Cirillo et al., 2009). However, the exact mechanism by which the ROS and nitric oxide inhibit the intracellular survival and replication of *M. tuberculosis* in activated macrophages is not fully understood (Chan et al., 2001, Venketaraman et al., 2003a).

1.6 .1 Reactive oxygen species

Reactive oxygen species (ROS) are important in innate immune responses against invading micro-organisms. ROS production increases rapidly in the early stages of intracellular infection

(Spooner and Yilmaz, 2011). Reactive oxidative intermediates (ROI) include the superoxide anion ($\text{O}_2^{\bullet-}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($\bullet\text{OH}$), and single oxygen ($^1\text{O}_2$) (Nathan and Ding, 2010). There are two main sources for ROS production in infected macrophages; nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), which is a membrane bound enzyme complex found within the plasma membrane and in the membrane of phagosomes, and the mitochondrial electron transport chain (Mt-ETC) (Spooner and Yilmaz, 2011, Koopman et al., 2010). The NADPH oxidase complex is the most common source for ROS production by phagocytic cells (Zalba et al., 2001). It is responsible for the transfer of electrons from cytoplasmic NADPH to O_2 in the membrane of phagosomes, the endosome and cell membrane (Leto et al., 2009). The NADPH complex is made up of different *phox* (PHOX for PHagocyte OXidase) subunits: the cytosolic p40^{phox}, p47^{phox}, and p67^{phox}, besides the cell membrane-bound phagocytic oxidases p22^{phox} and gp91. Subunits p22^{phox} and gp91^{phox} are the superoxide-generating core of NADPH oxidase (Babior, 1999, Vignais, 2002). NADPH- dependent oxidase generates $\text{O}_2^{\bullet-}$ in phagocytic cells and form H_2O_2 in the presence of superoxide dismutase enzymes. Both H_2O_2 and $\text{O}_2^{\bullet-}$ are highly toxic and can destroy biomolecules (Anjem et al., 2009).

The role of reactive oxygen species in intracellular survival of mycobacterial species is unclear. ROS are extremely toxic to *M. tuberculosis* (Edwards et al., 2001). Endothelial cells produce ROS in response to the mycobacterium infection, and a correlation was

found between a high ROS production and mycobacterium elimination (Garcia-Perez et al., 2011). There is also an increased susceptibility to mycobacterial infection in individuals with NADPH oxidase defects (Lau et al., 1998). However, while p47-*phox*^{-/-} mice have been reported to be very sensitive to *M. tuberculosis* infection (Cooper et al., 2000) and NADPH oxidase 2 (Nox2) knockout mice developed a more severe form of chronic granulomatous disease (CGD) due to BCG infection than wild-type mice (Deffert et al., 2014). The same correlation could not be made with *M. tuberculosis*, where there were no obvious significant effect of ROS on the intracellular survival in either *in vitro* and *in vivo* murine studies (Chan et al., 1992, Cooper et al., 2000).

Instead of suppression of bacterial growth, ROS have been seen to help *M. tuberculosis* and *M. abscessus* growth in infected macrophages (Oberley-Deegan et al., 2010a, Oberley-Deegan et al., 2009). Voskuil et al. (2011) reported that the resistance of *M. tuberculosis* to reactive oxygen species increased overwhelmingly when the bacteria were exposed to increasing doses of ROS, they also found that different oxidative stress defence genes were expressed by the bacteria in response to the ROS. In contrast to ROS, antioxidants have been reported to play important roles in limiting intracellular survival of mycobacteria in macrophages through inducing cell apoptosis (Venketaraman et al., 2003a). Antioxidants, such as MnTE-2-PyP or *N*-acetyl-*L*-cysteine (NAC), have been reported to inhibit intracellular growth of *M. abscessus* in

human monocyte-derived macrophages (Oberley-Deegan et al., 2010a). Glutathione has been found to interact with nitric oxide to produce a more stable form of nitric oxide known as S-nitrosoglutathione and both glutathione and S-nitrosoglutathione are bactericidal (Deneke and Fanburg, 1989, Jagannath et al., 1998, Morris and Hansen, 1981). The interactions between ROS and nitric oxide are considered to be essential for killing intracellular mycobacteria. Superoxide and nitric oxide are found to react and form a very toxic radical known as a Peroxynitrite (ONOO⁻) (Deffert et al., 2014).

1.6.2 Nitric oxide

Nitric oxide is an important intracellular molecule with a number of functions ranging from neural communication, vasodilation and is an important innate antimicrobial agent (Moncada et al., 1991). In macrophages activated by lipopolysaccharide (LPS), released nitric oxide is formed by oxidation of the terminal guanidino nitrogen atom of *L*-arginine catalysed by the NADPH-dependent enzyme inducible nitric oxide synthase (iNOS) (Kirikae et al., 1996, Long et al., 1999). In murine macrophages, the subcellular localized iNOS is found in two forms, a cytosolic form and vesicular form (Vodovotz et al., 1995). Kirikae et al. (1996) found about a half of iNOS activity and iNOS protein to be vesicular (Kirikae et al., 1996). Nitric oxide is a highly reactive and diffusible free radical and is soluble in both water and lipids (MacMicking et al., 1997). The molecule reacts with

oxygen and ROI to form more stable and toxic products such as nitrogen oxide, nitrate, dinitrogen trioxide, and peroxyxynitrite (Zhu et al., 1992, MacMicking et al., 1997). Nitric oxide is bactericidal; a concentration of less than 100ppm kills more than 99% of cells in a *M. tuberculosis* culture (Long et al., 1999, Lowenstein and Padalko, 2004). Nitric oxide plays essential roles in killing of *M. tuberculosis* and *M. bovis* BCG in murine macrophages (Jagannath et al., 1998, Long et al., 1999, MacMicking et al., 1997, Chan et al., 1995, Ling et al., 2013). Nitric oxide production is the first killing mechanism involved in *M. bovis* infection in murine J774 macrophages (Anes et al., 2006). The exact mechanism by which nitric oxide and its products kill *M. tuberculosis* is unknown but it has been suggested to disrupt the bacterial DNA, proteins, signalling, leading to induction of apoptosis of mycobacterial infected cells (Chan et al., 2001).

In contrast to the significant roles of nitric oxide in killing *M. tuberculosis* by murine macrophages, in human models of *M. tuberculosis* infection, the bactericidal role of nitric oxide and its products is still controversial (Nathan and Shiloh, 2000). In *M. tuberculosis* infection of human macrophages nitric oxide is not important for suppression of the bacterial growth in early infection (Jung et al., 2013). Also it has been found that IFN- γ -stimulated human alveolar macrophages failed to inhibit bacterial growth (Aston et al., 1998). Human macrophages and epithelial cells produce nitric oxide in response to *M. tuberculosis* infection (Rich et

al., 1997, Rockett et al., 1998). Moreover, Nozaki et al. (1997) reported that human alveolar macrophages are efficient in killing *M. bovis* BCG which was dependent on nitric oxide and its reactive products together with the oxygen radical, peroxynitrite.

Some mycobacterial species, especially *M. tuberculosis*, have evolved different ways to resist the toxic effects of nitric oxide (Chan et al., 2001). For example, the *noxR1* gene has been reported to play an important role in the bacterial resistance (Ehrt et al., 1997). The *noxR3* gene has also been reported to protect *M. tuberculosis* from nitric oxide and ROS (Ruan et al., 1999). Furthermore, in murine macrophages, *M. tuberculosis* inhibits the delivery of iNOS to phagosomes by interfering with the scaffolding protein EBP50 which is normally associated with iNOS and binds it to the cell cytoskeleton, actin (Davis et al., 2007). Miller et al. (2004) stated that the recruitment of iNOS to infected phagosomes is actin-dependent.

Mammalian macrophages have been widely used as a model system to study the effects of innate antimicrobial mechanisms on *M. tuberculosis* and *M. bovis* BCG. Nitric oxide is found to play an important role in inhibiting bacterial growth, in contrast to ROS roles which are not fully understood. In the context of *M. avium* infection of chicken cells, nothing is known about chicken macrophages as a model system to the study of *M. avium*.

1.7 Intracellular survival of mycobacteria

The kinetics of the intracellular survival of *M. tuberculosis* inside the phagocytic cells correlates with invasion mechanisms. Pathogenic mycobacteria have developed a number of mechanisms to avoid the destructive environment of phagocytic cells. Such mechanisms include invasion the host cells via specific receptor (Tessema et al., 2001b), disruption of the cell cytoskeleton (Hestvik et al., 2005), release from the phagocytic vacuoles to the cell cytosol (Stamm et al., 2003), inhibition of phagosomes acidification (Meena, 2010), interfering with phagolysosomes maturation (Vergne *et al.*, 2003), protection against oxidative radicals (Yuan et al., 1995, Dussurget et al., 2001) and inhibition of the macrophage response to IFN- γ myeloid differentiation factor 88 (MyD88) –dependent and -independent mechanisms (Fortune et al., 2004).

Invasion via a specific receptor could have significant impact on mycobacterial survival within the infected macrophages. For instance, FC receptor-mediated phagocytosis of particles induces a respiratory burst and inflammatory response in macrophages (Aderem and Underhill, 1999, Ernst, 1998). In contrast, invasion via CR3 receptors prevents the activation of the macrophages (Caron and Hall, 1998). In the context of the interaction between *M. avium* and chicken macrophages, the role of complement-, mannose- and scavenger receptors in invasion of avian and human cells by *M. avium* is not clear. Understanding the role of the receptor-mediated

phagocytosis will be valuable in assessing intracellular survival of *M. avium*.

Another intracellular survival mechanism that can be utilized by intracellular pathogens includes disruption of host cell cytoskeleton, in eukaryotic cells; cytoskeleton is a vulnerable target for various intracellular pathogens during infections (Hestvik et al., 2005). It has been reported that pathogenic bacteria induce rearrangement of the cell cytoskeleton through secretion of effector proteins including, *Chlamydia* CopN (Alzhanov et al., 2009, Scidmore, 2011, Archuleta et al., 2011), *Edwardsiella tarda* EseG (Xie et al., 2010) and *L. monocytogenes* ActA (Stavru et al., 2011). However, the phagocytosis is an actin dependent process; early formed vacuoles possess an actin coat and then the coat is subsequently removed through a myosin Va dependent mechanism (Al-Haddad et al., 2001). The trafficking of the formed vacuoles turns into a tubulin-dependent process (Blocker et al., 1998). Depolymerization of the tubulin network significantly reduces the contact and fusion of endocytic vacuoles (Blocker et al., 1997). It has been suggested that microtubules play potential roles in acquiring of Rab5 (early endosome marker) and Rab7 (late endosome marker) on the phagosomes and therefore maturation (Harrison and Grinstein, 2002).

Rearrangement of tubulin dynamics occurs as a results of effector proteins of pathogens on the tubulin subunit (Radhakrishnan and

Splitter, 2012). Intracellular pathogens such as *Listeria monocytogenes*, *M. marinum* and *Shigella flexneri* have been shown to release from the phagosomes to the cell cytosol and then spread between neighbouring cells during infection (Stamm et al., 2003). It has also been reported that *M. tuberculosis* H37Rv (virulent strain) releases to the cell cytosol and replicates there (van der Wel et al., 2007) whereas, BCG vaccine and non-virulent strain of *M. tuberculosis* H37Ra remains in phagosomes (McDonough et al., 1993). Virulent strains of *M. tuberculosis* secrete ESX-1 secreted protein ESAT-6, a membrane-lysing protein to induce rupture of the phagocytic vacuoles and access to the cytoplasm (Simeone et al., 2012).

In murine macrophages it has been shown that nearly 85% of *M. bovis* BCG vaccine and *M. tuberculosis* reside within the fused vacuoles (phagolysosomes) 2h post-infection. However the level of fusion of vacuoles containing *M. tuberculosis* H37Rv and H37Ra is much reduced with only 50% fusion levels 96 hours post-infection (McDonough et al., 1993). The fusion levels of BCG vaccine and of heat-killed H37Rv and H37Ra remained unchanged suggesting that the increases in the number of the non-fused vacuoles is a result of an active process on the part of H37Rv. Releasing the bacteria from fused vacuoles to cytosol and replication of the bacteria in the non-fused phagosomes thus aids survival (McDonough et al., 1993).

Mycobacteria within the phagocytic vacuoles of infected macrophages encounter an acidic environment (Piddington et al., 2000). Macrophages offer very stressful conditions to the intracellular pathogens through induction of acidic environment (Booth, 1985). Under low pH conditions enzymes and proteins are not working normally, the bacteria try to keep their environmental pH close to 7 (Booth, 1985). It has been seen that *M. tuberculosis* is very sensitive to environmental pH; the bacterial growth was restricted at pH 6.0, whereas, *M. marinum*, *M. avium*, and *M. chelonae* grow well at pH 6.0 (Piddington et al., 2000). However, the survival mechanisms of *M. tuberculosis* and *M. bovis* BCG within the mammalian cells are not fully understood. To our knowledge no study yet has been done to understand the intracellular localization and trafficking of *M. avium* within mammalian and avian macrophages.

The ingested pathogens are exposed to an extensive array of chemicals including reactive oxygen and nitrogen radicals. Pathogenic mycobacteria like the Erdman strain of *M. tuberculosis* have been found to modify their mycolic acids by cyclopropanation, leading to significantly reduced bacterial susceptibility to peroxide (Yuan et al., 1995). Like other bacteria, mycobacteria have developed systems to resist reactive oxygen species (ROS); the systems include different enzymes such as glutathione peroxidase, glutathione reductase, copper and zinc-dependent superoxide dismutase (Cu-ZnSOD) and catalase and manganese-dependent

superoxide dismutase (MnSOD) (McCann et al., 2007). The mycobacterial genome contains several *secA* genes (*secA1*, *secA2*) (Braunstein et al., 2003). These genes are responsible for secretion of superoxide dismutase (SodA) and catalase-peroxidase (KatG) which are essential for detoxification of ROS (Braunstein et al., 2003, Rouse et al., 1996). In addition, the peroxynitrite is destroyed by alkylhydroperoxide reductase AHC (Bryk et al., 2000). TACO proteins (tryptophan-aspartate containing coat protein) are secreted into the phagosome by pathogenic mycobacteria. These proteins are linked to the cell wall synthesis for cellular homeostasis and energy production, and protect mycobacteria from oxidative damage of reactive nitrogen species and reactive oxygen species inside the phagosome (Meena and Rajni, 2010, McCann et al., 2007).

Furthermore, *M. tuberculosis* has been shown to alter the pro-inflammatory cytokines at the transcriptional level, including IFN- γ due to the interaction of the 19-kDa lipoprotein and mycolyl arabinogalactan peptidoglycan complex (mAGP). The 19-kDa lipoprotein inhibits IFN- γ signalling in a Toll-like receptor (TLR) 2-dependent and Myd88-dependent fashion (Fortune et al., 2004). *M. tuberculosis* can also suppress the expression of MHC class II on macrophages to prevent antigen presentation to CD4⁺ T cells by blocking transport and processing of the molecules, thereby avoiding an IFN- γ response (Hestvik et al., 2005).

1.8 Aims of the study

Taken together, data from previous studies on the interactions between *M. tuberculosis* and host cells indicate that mycobacteria enter the host cells via different cell receptors. It would be predicted that intracellular survival of the mycobacteria will depend on the invasion mechanisms utilized. The innate antimicrobial response will play a crucial role in the early stages of mycobacterial infection. This study investigated the hypothesis that *M. avium* uses the equivalent mechanisms to invade and survive in avian cells as *M. tuberculosis* and *M. bovis* do in mammalian cells. The work presented describes the mechanisms of invasion, looking at the role of different host sera, receptors and cellular scaffolding and discussed the intracellular survival kinetic and importance of innate antimicrobial mechanisms in bacterial survival.

2.0 General materials and methods

2.1 Media

7H10 agar (Difco, USA) was used for routine cultivation of mycobacteria and determination of intracellular bacterial counts from infected tissue culture cells. Middlebrook 7H9 broth (Difco) was prepared and used for growing mycobacteria prior to experimental infection of cultured cells. Media were prepared according to the manufacturer's instructions and sterilized by autoclaving at 121°C for 15 min, the autoclaved agars were allowed to cool down to nearly about 60°C and supplemented with 10% of oleic acid albumin dextrose catalase (OADC) supplement which is composed from the following ingredients (oleic acid 0.025 g, bovine albumin fraction V 2.50 g, dextrose 1.00 g, catalase 0.002 g, sodium chloride 0.425 g and distilled water 50 mL) (Difco) and 0.5% v/v glycerol (Fisher Scientific, UK).

2.2 Mycobacterial strains

The five strains of *M. avium* sub species *avium* used in this study were *M. avium* NC08562.02, *M. avium* NC08559-07, 61-3623-05, 61-4627.08 and 61-3728.08, which are referred to as Ma1, Ma2, Ma3, Ma4 and Ma5 in all figures, respectively. Ma1 and Ma2 were isolated from diseased chickens in the 1960s and Ma3, Ma4 and Ma5 were isolated from cattle and were a gift of Dr C. Wray, Animal and

Plant Health Agency, Weybridge, UK. The bacterial strains have been grown in 7H9 broth under aerobic conditions at 37°C. The cultures were incubated in a shaker incubator (Forma orbital shaker-Thermo, UK) at 150 revolutions per minute (rpm). The bacteria were grown to a concentration of approximately 10^8 bacteria per ml depending on the optical density. The challenge doses of the bacterial strains of 5×10^6 CFU/ml were determined using 1 ml of the bacterial suspension which was transferred in a 15 ml centrifuge tube and centrifuged at 1000x g for 10 min. The supernatant was discarded and the cell pellet was re-suspended in PBS according to the required challenge dose which was calculated by measuring the optical density of bacterial cultures at 600 nm by spectrophotometer (Libra S22 biochrom, Scientific Laboratory suppliers, UK) and comparing the bacterial CFU number were determined by using the Miles and Misra method (Miles et al., 1938).

2.3 Culture of chicken macrophages (HD11)

HD11 cells (Beug et al., 1979) were grown in T-75 flasks (Thermo Fisher Scientific, Roskilde, Denmark) in RPMI 1640 medium containing 200 mM L-glutamine (Life Technologies), 5% v/v foetal calf serum (FCS), 5% v/v chicken serum, and 10% v/v tryptose phosphate broth (Sigma, England). The cultures were split every 3 days, the old medium was removed and the flasks were washed twice with 5 ml of 1% phosphate buffer saline PBS (Gibco) at 37°C to remove any unattached cells from the flask. 5 ml of trypsin (10

mM)- PBS 10% v/v was added to the cells and flasks were then incubated at 37°C for 5 min until the cells were detached from the flasks. 5 ml of the complete medium was added to the detached cells and transferred to sterile 15 ml centrifuge tubes (Thermo Fisher Scientific) and pelleted at 300x g for 5 min, the supernatant was discarded and cells were re-suspended thoroughly using 5 ml of the complete medium. The cell viability was > 95% by trypan-blue exclusion (Sigma). To each well of a 24-well plate, 1 ml of cell suspension was added and incubated to obtain 5×10^5 cells / well as determined by cell count using a haematocytometer. Maintenance flasks and seeded plates were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

For storage, the cells were collected from maintenance flask as mentioned above. The cells were centrifuged at 300x g for 5 minutes and counted for freezing. The pellet was re-suspended in a suspension made of 90% FCS and 10% of dimethyl sulfoxide (DMSO) (Fisher Scientific, UK) as cryopreservative medium and one ml containing 1×10^6 cells were transferred into pre-chilled cryovials which were labelled and stored in -80°C for 24h before were transferred to liquid nitrogen. To revive cells, vials were thawed and the content was washed and then transferred to a flask containing the culture media. The flask was then incubated to allow cell growth.

2.4 THP-1 cells

Frozen THP-1 cell (Tsuchiya et al., 1980) stocks were thawed at 37°C and diluted in 5 ml of complete RPMI medium (Gibco) containing 200 mM L-glutamine, 10% v/v FCS to dilute DMSO in which the cells were frozen. The cells were pelleted by low speed centrifugation at 1000x g for 5 minutes and then resuspended in 10 ml complete RPMI medium and aliquoted into cell culture flasks to be incubated at 37°C in a humidified atmosphere containing 5% CO₂. Two days prior to infection, the cells were harvested, washed and resuspended in fresh medium. To induce differentiation into adherent macrophage-like cells, phorbol-12-myristate-13-acetate (PMA)(Sigma, UK) was added at 50 ng/ml, and the cells were seeded into culture wells of 1.91 cm² at a concentration of 5×10^5 cells/ml. Seeded cells were incubated for 48h at 37°C in a humidified atmosphere containing 5% CO₂ prior the infection.

2.5 Gentamicin protection assay.

For *in vitro* infection experiments, mycobacteria were grown to exponential growth phase which were centrifuged at 1000xg for 10 min at 20°C prior to re-suspension in PBS according to the required challenge doses which were calculated by measuring the optical density of bacterial growth at 600 nm and comparing with the bacterial CFUs number determined by using the Miles and Misra method. Infections were carried out using a multiplicity of infection

(MOI) of 10:1 (10 bacteria to 1 cell) (Kaiser et al., 2000) and tissue culture cells were then incubated for 48h at 37°C. Intracellular bacterial counts were determined at different time points 2h, 6h, 24h, and 48h post-infection. Intracellular counts were detected after lysing cells with 1% Triton X-100 solution (Fisher Scientific, UK). Cell supernatants were kept at -20°C for the estimation of nitric oxide and lactate dehydrogenase (LDH).

The invasion and persistence of the strains of mycobacteria used within THP-1 and HD11 cells was determined by using the gentamicin protection assay as previously described by Jones et al. (2001) and Smith et al. (2005). Prior to using the gentamicin protection assay, the sensitivity to gentamicin of *M. avium* isolates was evaluated using the disc diffusion method and dilution methods. Cells were seeded in 24 well plates, HD11 were left for 24h and THP-1 for 48h in the presence PMA (phorbol-12-myristate-13-acetate) (50 ng/ml) to differentiate THP-1 to macrophages. Cells were infected with different mycobacterial strains at MOI 10:1. After 1h of incubating the bacteria with the cells, the medium was removed and the cells were then washed three times with 1 ml of PBS at 37°C. The medium was changed to RPMI containing gentamicin sulphate (100 µg/ml) (Sigma) and further incubated for another 1h to kill extracellular bacteria. The cells were then washed three times with 1 ml of PBS at 37°C. The count of bacterial invasion and / or uptake was made by adding of 200 µl of 1% Triton X-100 in PBS (for lysing cells) and then plating on 7H10 plates for

counting colony forming units (CFUs) of the bacteria. To determine bacterial survival inside infected cells, cells were kept in complete media containing gentamicin sulphate (100 µg/ml) to inhibit the extracellular bacterial multiplication. At 6h, 24h and 48h post-infection, the cell monolayer was washed three times in pre-warmed PBS before lysing cells with 200 µl of 1% Triton X-100 solution. Viable counts were determined and are shown as CFU / ml.

2.6 Determination of intracellular bacteria by microscopy.

The number of bacteria in individual cells was determined as follows. Cells were seeded and infected exactly as described above but with wells containing 13-mm plastic coverslips (Thermanox, USA). To determine the number of intracellular bacteria, cell monolayers were washed in PBS and fixed in 4% v/v paraformaldehyde for 20 min at room temperature. The cells were permeabilized with 0.05% Triton X-100 in PBS for 15 min. The infected cells were then stained by flooding with auramine O-rhodamine for 15 min, rinsed in water and flooded with acid alcohol (0.5 ml of concentrated HCl in 100 ml of 70% v/v ethanol) for 2 min. Finally the cells were washed and mounted on glass slides using Vectashield (Vectors laboratories, USA) antifade reagent containing the nuclear counterstain DAPI (Life Technologies). Cells were visualized and intracellular bacteria were enumerated using a LEICA DM 5000B fluorescence microscope with a 63 X 1.25 lens. To determine the number of bacteria per cell, 30

fields on each coverslips were observed and the infected cells were enumerated at each time point. The quantitative evaluation of intracellular pathogens by the AO staining protocol yields bright green coloured bacilli within a blue background of DAPI stained cells (See appendix figure 8.3).

2.7 Role of sera on invasion rate

To study the role of different sera in mycobacterial invasion, the gentamicin protection assay was used as previously described. The monolayers were washed three times with PBS at 37°C; the experiments were divided into four groups. In the first group, RPMI 1640 was supplemented with 10% v/v of FCS (VWR International Ltd/ UK), in the second group, RPMI supplemented with 10% v/v of chicken serum (Sigma-Aldrich/ USA), 10% of human serum (MP Biomedicals, LLC/ UK) was used in the third group, while serum free RPMI was used in the fourth control group. The infections were carried out with MOI of 10:1 as mentioned before; all plates were incubated at 37°C under 5% of CO₂ and humid conditions. After 1h of incubating the bacteria with the cells, the cells were washed three times with 1 ml of PBS at 37°C and the medium was changed with RPMI containing gentamicin sulphate (100 µg/ml; Sigma). The plates were then incubated for another 1 hour to kill extracellular bacteria. The cells were then washed and lysed for bacterial counts as described above.

To investigate the interaction between different host sera and their effects on the bacterial phagocytosis by the host cells, the gentamicin protection assay was used and infections were carried out with MOI of 10:1 as mentioned before; the experiments were divided into six groups. Non-treated host sera were used during THP-1 and HD11 cell infections. In the first group, RPMI 1640 was supplemented with 10% v/v of FCS, in the second group, RPMI supplemented with 10% v/v of chicken serum, 10% of human serum was used in the third group, while serum free RPMI was used in the fourth groups, 10% FCS+10% HS were used in fifth group and in the sixth group 10% CHS+10%HS were used. After 1h of incubating the bacteria with the cells, the cells were washed three times with 1 ml of PBS at 37°C and the medium was changed with RPMI containing gentamicin sulphate (100 µg/ml; Sigma). Then the bacterial counts were determined as described above.

To detect any variation between the role of fresh and frozen serum stored at -20°C on opsonisation and phagocytosis of *M. avium* by the host cells, the gentamicin protection assay was used and infections were carried using RPMI containing 10% of fresh host sera compared to RPMI containing 10% frozen sera. Fresh chicken serum was collected from females chicks (Harlan Laboratories U.K Ltd) and fresh calf serum was collected from young female of two months old (Sera Laboratories international Ltd, UK). Fresh human serum was donated by a PhD student at the School of Life sciences,

University of Nottingham. The fresh sera were delivered on dry ice. All experiments were run on the delivery days.

To determine the role of host serum complement on the bacterial invasion in both cell lines, firstly, stock solutions of chelating agent EGTA (ethyleneglycol-bis (beta-amino-ethyl ether) *N, N'*-tetraacetic acid, Sigma, USA) (inactivates the classical pathway of the complement system) and EDTA (disodium ethylene diamine tetra acetate, Fisher Scientific / UK) (inactivates the complement system in both classical and alternative pathways) were prepared in normal saline as mentioned by (Fine et al., 1972). 100 mM of stock solutions were prepared by dissolving 5g of EDTA (molecular weight 292.24) in 134.3 ml of normal saline, while 5g of EGTA (molecular weight 380.35) was dissolved in 131.5 ml of normal saline; the mixtures were heated at 60°C to dissolve the powder with drop wise adding of 5N NaOH. The stock solution of 100 mM $MgCl_2$ was prepared by also dissolving of 3g of $MgCl_2$ (molecular weight 203.3) powder in 147.5 ml of normal saline. The pH of all solutions was changed back to 7.4 by using 1 N HCl and 5 N NaOH. All the prepared stock solutions were kept in cold room at 4°C. Then the host sera were prepared; in all experiments, the chelating agents were added at concentration of 0.1ml of stock solution (100 mM) to 0.9 ml of undiluted sera to make a final concentration of 10 mM, the well mixed mixtures were incubated for 1h at 37°C in incubator. The heat inactivated sera were prepared by incubating sera in a water bath at 56°C for 1h.

To avoid detachment of HD11 cells, fibronectin coated plates were used. 24 wells plates were coated with 200 µl of fibronectin (Sigma. USA) at concentration of 50 µg/ml according to the manufacturer's instructions. The coated plates were then incubated at 37°C overnight. On the next day the fibronectin solution residues were removed and the coated wells were allowed to dry in a sterile hood for 20 min. 5×10^5 HD11 cells were seeded in triplicate wells and seeded plates were incubated overnight to be ready for the next day.

To detect the ability of *M. avium* Ma1-5 to activate the complement system by both classical and alternative pathways and to determine the effect of the complement components on the opsonisation and phagocytosis of *M. avium* by the host cells the treated host sera were used at a concentration of 10% v/v in RPMI and compared with controls consisting of serum-free RPMI (negative control) and 10% v/v normal untreated host sera in RPMI as can be seen in the experimental design (Figure 2.1). Gentamicin protection assay was used and the infections in both cell lines were carried out at MOI 10:1 under the presence of treated and non-treated host sera and 10 mM $MgCl_2$ in HD11 cells, but not THP-1. Following infection, the cell monolayers were washed three times in pre-warmed PBS before lysing cells with 200 µl of 1% Triton X-100 solution. Viable counts were determined and are shown as CFU/ml.

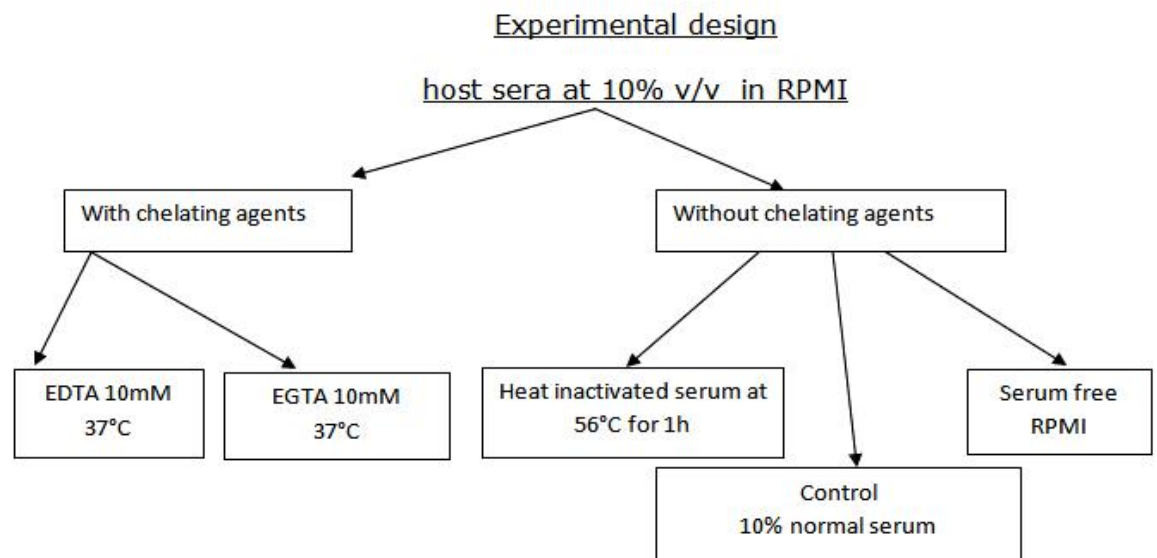


Figure 2.1: Basic experimental design to detect the role of host serum complement in opsonisation and subsequent invasion of *M. avium* of THP-1 and HD11 cells. Positive control 10% normal host serum. Negative control serum free and no chelators. Treatments, EGTA treatment 10 mM with host serum at 10%, EDTA treatment 10 mM with host serum at 10% and heat inactivated serum with no chelators (HIS).

2.8 Treatment of cells

2.8.1 Treatment with ROS and nitric oxide stimulators

Specific stimulators were used when required to trigger THP-1 and HD11 cells to produce more ROS and nitric oxide production in response to *M. avium* infection. For ROS production, Phorbol-12-myristate-13-acetate (PMA) (Sigma) was used at a final concentration of 100 ng/ml to activate THP-1 cells for 48h and HD11 cells for 24h. For nitric oxide production, HD11 cells were incubated

with recombinant chicken IFN- γ / 100 U/ml (Kingfisher Biotech, INC. USA) for two hours. Following the incubation period, the cells were then washed and prepared for the bacterial infection using the gentamicin protection assay as described above. The concentrations of these two stimulators used in this study were optimized and selected according to the ROS and nitric oxide levels produced and their cytotoxicity levels (data shown in section 8.4 and 8.5).

2.8.2 Treatment with ROS and nitric oxide inhibitors

When needed, the cells were treated with specific inhibitors; these inhibitors were added from 0 to 2h before challenging THP-1 and HD11 cells with *M. avium*. The inhibitors used were *N*-acetyl-L-cysteine (NAC, 10mM) and diphenyleneiodonium chloride (DPI, 10 μ M) (MP Biomedicals, LLc, UK) for inhibition of ROS production. The inhibitors were added before the infection for two hours and maintained throughout the experiments. *NG*-Monomethyl-L-arginine, monoacetate *L*-NMMA (Sigma, UK) was used for inhibition of iNOS. *L*-NMMA was added to HD11 cells, to a final concentration of 1 mM prior to addition of the mycobacterial strains, and was maintained in the tissue culture (HD11) after initial infection and throughout complete experiments. For cytoskeleton disruption, THP-1 and HD11 cells were incubated with actin inhibitor cytochalasin D (CD, 1 μ g/ml) (MP Biomedical, LLC. France) and the tubulin disruptor nocodazole (Noco, 20 μ M) (VWR International LTD. UK) for

45 min before bacterial infection and the inhibitors were maintained throughout the experiment over 48h.

For examining the role of cell surface receptors in invasion of *M. avium* to THP-1 and HD11 cells, different soluble chemical ligands and the gentamicin protection assay were used. The ligands used were heparin sodium salt from porcine intestinal mucosa (Sigma, USA) at a concentration of 10 U/ml for blocking complement receptors 3 CR3 (CD11b/CD18) (Peter et al., 1999, Diamond et al., 1995). Dextran sulfate sodium salt (Sigma, Denmark) was used at a concentration of 100ug/ml for blocking scavenger receptors (SRs) (Olsson and Oldenborg, 2008, Tsubamotoa et al., 1994). Mannose receptors (MRs) were blocked by using water soluble sugars such as α -methyl *D*-mannoside (Sigma, Germany) at a final concentration of 2% (Bermudez et al., 1991, Ampel et al., 2005).

To detect the effect of the cytoskeleton on invasion rates, THP-1 and HD11 monolayers were incubated either with cytochalasin D, 1ug/ml, nocodazole, 20 μ M alone or simultaneously for 45 min before challenging with *M. avium* strains. Controls were the HD11 and THP-1 infections in the absence of any blocker.

To detect the interactions between mannose-, scavenger- and complement receptors and cell cytoskeleton, actin and tubulin, the seeded monolayer cells were washed and prepared for the gentamicin protection assay as described above. The experiments were grouped into different groups (Table 2.1). In the first two

mannose receptors and scavenger receptors groups, serum free RPMI was used, whereas, in the CR3 group, RPMI was supplemented with 10% v/v of FCS for HD11 cells and 10% v/v of human serum was used for THP-1 cells. Triplicate wells were used for each strain of *M. avium* in each HD11 and THP-1 group tested. For the interaction between mannose receptor and the cytoskeleton, triplicate wells were used for blocking mannose receptors alone and same number were used for each of cytochalasin D and nocodazole treated cells, plus three wells for mannose receptors blocker together with cytochalasin D and three wells for mannose receptors together with nocodazole. Three wells in the last row were allocated for non-treated cells as a control. Similarly, the same technique was used to study the interactions between scavenger receptors and complement receptors CR3 and the cell cytoskeleton of both THP-1 and HD11.

Table 2.1: Experimental design for examining the interaction between receptors and cytoskeleton.

Receptors	Receptor blockers	Actin disr	Tubulin disr	Rec. B +Actin dis.	Rec. B +Tub. Dis.	control
Mannose receptor (MR)	Mannose	CD	Nocodazole	CD + Mannose	Nocodazole + Mannose	No treated cells as basic invasion
Scavenger receptor	Dextran	CD	Nocodazole	CD + Dextran	Nocodazole + Dextran	
Complement receptor 3 (CR3)	Heparin	CD	Nocodazole	CD + Heparin	Nocodazole + Heparin	

2.9 Measurement of reactive oxygen species ROS.

The production of reactive oxygen species by infected cells was determined by using the nitroblue tetrazolium (NBT) reduction assay

(Segal, 1974). To detect reactive oxygen species in mycobacterial infected THP-1 cell and HD11 lines, infection of cells were carried out as described above in 96 wells cell culture plates at MOI of 10:1. At each time point, the supernatants were discarded and the cells were incubated with 50ul of a 1 mg/ml NBT solution made in PBS. After 45 min the reaction was stopped with 100 ul of 1 N hydrochloric acid and the plates were shaken by using a shaker (Heathrow Scientific Sea Star version 1.11) at 60 rpm for 10 min at 20°C. The wells were then washed with PBS at 37°C and shaken again for 10 min at 20°C. The washed cells were then re-suspended in 150 µl of DMSO (Sigma, USA) and 10ul of 1N sodium hydroxide was added to generate the colour. The optical densities of the reactions were determined by measuring the absorbance at 630 nm using a micro plate reader (Microplate absorbance reader, LT-4000, Labtech, Sussex, and UK). Positive controls of HD11 and THP-1 cells were treated with 10 ul of LPS at 50 µg/ml as a final concentration and negative controls wells of mock-infected (PBS). Samples were done in triplicate within the experiment and each experiment was done in triplicate.

2.10 Greiss assay

To estimate nitric oxide (NO) production by the THP-1 and HD11 macrophages in response to the mycobacterial strains, nitrite was determined (a representative for nitric oxide production) by testing the supernatant of the infected tissue culture at various times using

the Griess assay (Promega, Madison, USA) (Green et al., 1982). Briefly, 50 µl of the culture supernatants from each sample, stored at -20°C and thawed, was transferred to a 96-well flat-bottom microtitre plate (Thermo Fisher Scientific) before adding the same volume of sulfanilamide (1% sulfanilamide in 5% phosphoric acid) solution to each well. The plate was then incubated for 10 min at room temperature in a dark place followed by adding the same volume of NED (0.1% *N*-1-naphthylethylenediamine dihydrochloride in water) solution and incubated for other 10 min. The concentration of nitrite was determined by measuring the absorbance at 540 nm using a micro plate reader (Microplate absorbance reader, LT-4000, Labtech, Sussex, UK). For each assay a standard reference curve of nitrite was prepared in order to accurately measure nitrite concentration (See appendix figure 8.2). For that purpose seven points of a twofold serial dilution were used in triplicate. Three wells were used for each triplicate sample for each time point to obtain a precise reading for the reaction. For controls, supernatants of cells that were challenged with 100 µl of LPS at 50 µg/ml and 100 µl of PBS were used for positive and negative control, respectively.

2.11 Cytotoxicity assay

To determine the cytotoxic effect of *M. avium* Ma1-5 on human (THP-1) and avian (HD11) cells, aliquots of the stored supernatants at -20°C were used to determine the time-course liberation of lactate dehydrogenase (LDH) (a cytoplasmic enzyme which is

released as a result of damage of plasma membrane and cell death) to the culture medium at 2h, 6h, 24h and 48h after infection by using the CytoTox 96 kit (Promega, UK), according to the manufacturer's instructions. Cytotoxicity was expressed as a percentage, taking 100% as the activity of LDH released from cells lysed were prepared through three sequential freezing / thawing cycles, where the mock-infected cells were kept at -80°C for 30 min and followed by thawing at 37°C for 20 min. The percentage of the released LDH was determined by measuring the optical density at 490 and converted to percentage using the following formula: % of cytotoxicity = ((experimental release-spontaneous release- effectors rerelease) / (maximum release-spontaneous release)) X 100. The experiments were done in triplicate and the values were presented as means and standard errors. In all experiments the following controls were included: background (culture media alone), spontaneous LDH release (uninfected cells for spontaneous release of LDH), maximum LDH release (by incubating cells at -80°C for 30 minutes followed by thawing at 37°C for 15 min), effectors release (*M. avium* strains alone).

2.12 Role of ROS in intracellular survival of *M. avium* in THP-1 and HD11 cells.

To study the role of ROS in *M. avium* growth in THP-1 and HD11 cells specific stimulators were used to trigger THP-1 and HD11 cells to produce more ROS in response to *M. avium* infection comparing

to the inhibitor and non-treated control group. For ROS stimulation, Phorbol-12-myristate-13-acetate (PMA) (Sigma) was used at a final concentration of (100 ng/ml) to activate THP-1 for 48h and HD11 for 24h. Following incubating the cells with the stimulators, the cells were washed and prepared for the bacterial infection using the gentamicin protection assay as described above. For ROS inhibition, the cells were treated with specific inhibitors, which were added for 2h before uptake, and left throughout the experiment. The inhibitors used for inhibition of ROS production by *M. avium*-infected THP-1 and HD11 cells were *N*-acetyl-*L*-cysteine (NAC, 10mM) and diphenyleneiodonium chloride (DPI, 10uM) (MP Biomedicals, LLC, UK). At each time point, intracellular bacteria were recovered by lysing the infected macrophages as described above.

2.13 Effect of nitric oxide on intracellular survival of *M. avium* in HD11 cells.

To assess the involvement of nitric oxide in intracellular survival of *M. avium* strains, HD11 cells were treated with nitric oxide stimulator (rchIFN- γ) and inhibitor (*N*^G-Monomethyl-*L*-arginine, monoacetate *L*-NMMA) (Sigma) and compared with the non-treated cells. In the stimulated groups, HD11 cells were incubated for 2h with rchIFN- γ 100 U/ml followed by washing three times with PBS before infection. In the inhibitor groups, *L*-NMMA (an arginine analogue which is able to inhibit nitric oxide production) was added to HD11 cells, to a final concentration of 1 mM prior to addition of

the mycobacterial strains. The presence of *L*-NMMA was maintained in the tissue culture after initial infection and throughout the experiment. The control groups involved infection, where the cells were infected without any treatment, and with recovery of intracellular bacteria as described before.

2.14 Measurement of phagosomal pH

2.14.1 Labeling of *M. avium* and Zymosan A with fluorescent probes

M. avium strains and Zymosan A (MP Biomedicals, LLC, UK) were labelled with fluorescent probes as follows. On the day of infection, 1 ml of 4×10^8 of *M. avium* Ma1-Ma5 at their Log-phase were transferred to 15 ml tubes and centrifuged at $1000 \times g$ for 10 min. The supernatant was discarded and the cells pellets were re-suspended in PBS. 1 ml of 4×10^8 of Zymosan A was incubated in foetal calf sera for 30 min at 37°C , washed three times and re-suspended in PBS. NHS-CF (5(6)-carboxyfluorescein *N*-hydroxysuccinimide ester) (Sigma-Aldrich, UK) and NHS-Rho (NHS-rhodamine) (Fisher scientific, UK) were used to label *M. avium* Ma1-5 and Zymosan A as previously described by Oh and Straubinger (1996). Fluorescent probes at concentration of (100 μl of each at 0.2 mg/ml in DMSO) were added to the bacterial strains and Zymosan A suspensions. NHS-Rho is pH resistant, whereas NHS-CF is not. The labelled suspensions were vortexed for 30 sec and incubated at 4°C in dark for 30 min; the reactions were stopped by

adding 100 μ l of Tris HCL (sigma) pH (8.3) at a final concentration of 100 mM and kept at 4°C for 15 min again, then the labelled suspensions were washed and diluted according to the challenge doses at MOI 10:1.

2.14.2 Environmental pH of vacuoles containing *M. avium*

To measure pH of phagosomes containing *M. avium* in infected HD11, experiments were run as described by (Li et al., 2005), 100 μ l of HD11 suspensions were seeded in 96-well flat-bottom plates (Thermo Fisher Scientific) at 5×10^5 cells / ml for overnight, the seeded cells then were washed three times with PBS at 37°C. The washed cells were challenged with CF-Rho labelled bacteria and Zymosan A at MOI 10:1 using a gentamicin protection assay. At different time points post-infection (2h, 6h, 24h and 48h); the cell supernatant was replaced by PBS. Measurements were taken at an excitation wave length of 544 nm and emission of 590 nm for NHS-Rho and an excitation wavelength of 485 nm and emission wavelength of 520 nm for NHS-CF using FLUOstar Optima (BMG Labtech). The fluorescence units of each well were used to analysis data. For each well, the phagosomal pH was determined according to the standard calibration curve based on the NHS-CF/ NHS-Rho ratio versus pH. After taking the fluorescent measurements and at the end of each time point, *in situ* calibration curves of the NHS-CF/NHS-Rho emission ratio versus pH were made by treating wells

with 100 µl of buffers of defined pH (4-8) for 1 h at 37°C. pH buffers were made up of 0.2% Triton X-100 in PBS- 130 mM KCl (Sigma)- 30 mM sodium acetate-acetic acid (sigma) and 10µM Nigericin sodium salt (Sigma-Aldrich) for pH (4 and 5) or 10µM of Nigericin sodium salt-130 mM KCl-30 mM Na₂HPO₄ (for pH 6,7 and 8).

2.15 Optimal pH for the *M. avium* growth

To determine the optimal pH for *M. avium* growth, 7H9 broth supplemented with OADC 10% v/v was used as previously explained by (Oh and Straubinger, 1996) with a little modification. The bacterial strains *M. avium* Ma1-Ma5 were grown to a concentration of approximately 4 x10⁸ bacteria per ml depending on the optical density at 600 nm. 500 µl of the cultures of the bacterial strains were transferred into 5 ml of 7H9 broths of defined pH (4-8) to give an optical density (OD=~0.05 - 0.06 at zero time). The cultures were incubated in a shaker incubator at 150 rpm at 37°C. After 24h and 48h of incubation, the bacterial growths were measured by taking their optical density at 600 nm using spectrophotometer (Libra S22 Biochrom, Scientific Laboratory suppliers, UK).

2.16 Intracellular trafficking of *M. avium* in HD11 compartments

For quantitative determination of *M. avium* localization in HD11 compartments, the gentamicin protection assay was used. HD11 cells were grown in 24 wells plates at 1x10⁶ cells / ml per well; seeded cells were challenged with 5 *M. avium* strains and Zymosan

A at MOI of 10:1 and tissue culture cells were then incubated for 24h at 37°C. At different time points (2h, 6h and 24h) post-infection, the supernatants were discarded and the monolayers were harvested by adding 500 µl of cold Accutase® solution (Sigma-Aldrich) at 4°C for 8 min, the cells were washed and fixed in 4% v/v paraformaldehyde for 10 min at 4°C. Cell permeabilization was achieved with 10 min treatment with 0.2% Triton X-100 in PBS at 4°C. After blocking with 3% bovine serum albumin (BSA) in PBS for 45 min, the infected HD11cells were then incubated with primary antibodies for 1h at 4°C. Rabbit anti-chicken polyclonal anti-EEA1 antibody- early endosomes marker (ab2900) (Abcam, UK), Rabbit anti-chicken RAB7 polyclonal CPBT-56441RC (Creative BioMart, USA), and Rabbit anti-chicken polyclonal anti-LAMP-1 antibody- lysosome marker ab24170 (Abcam) were used at final concentrations of 1 µg/ml. Cells then were washed and incubated with the secondary antibodies goat anti rabbit IgG H&L (FITC) AB6717 (Abcam) at a final concentration of 1:2000 for a further 1h at 4°C in a dark place. The labelled cells were washed and resuspended in PBS before flow cytometric analysis. After staining, 400 µl of cells suspensions were pipetted to flow cytometry test tubes (Sarstedt, Germany), one tube for each measurement, experiments were performed on a BD FACSCanto™ II flow cytometer. Histograms were drawn and data were analysed by the BD FACSDiva software. The gated populations were used to determine mean fluorescence intensity values. The mean values of

bacterium-treated cells were been subtracted from the mean fluorescence intensity values of cells incubated in the absence of bacteria and Zymosan A.

2.17 Immunofluorescence

For fluorescence microscopy, cells were grown and infected in the same way using the gentamicin protection assay as described above, but with the wells containing 13-mm plastic cover slips (Thermanox, USA). The monolayers were washed in PBS and fixed in 4% v/v paraformaldehyde for 15 min at room temperature. Cell permeabilization was achieved with 10 min treatment with 0.05% Triton X100 in PBS. Following blocking with 3% bovine serum albumin (BSA) in PBS for 45 min, cells were washed extensively with PBS three times. The cover slips were then incubated with primary antibodies for 1h at room temperature, followed by PBS washing and incubating with the secondary antibodies for a further 1h at room temperature. For actin labelling, Alexa Fluor™ 546 Phalloidin Invitrogen at a final concentration of 200 units/ml, which is about 6.6 μ M (Fisher scientific/ UK) was used for 30 min, then the cell's DNA was visualized by DAPI (4',6-diamidino-2-phenylindole) for 5 min. Finally the cells were washed and mounted on glass slides using Vectashield (Vectors laboratories, USA) anti-fade reagent containing the nuclear counterstain DAPI (Life Technologies).

For nitric oxide and ROS co-localization with *M. avium*, Ma1 and Ma4 were chosen and the infected HD11 and THP-1 cells at different time point post-infection were examined and the percentages of co-localized bacteria were determined by using a LEICA DM 5000B fluorescent microscope. The numbers of co-localized and non-co-localized bacteria in 50 fields in cover slips were counted in triplicate wells of duplicate experiments for each strain.

For immunofluorescence microscopy the following antibodies were used: rabbit anti-chicken tubulin polyclonal antibody ab11270 (Abcam / UK), rabbit anti-human tubulin polyclonal antibody ab59680 (Abcam), rabbit polyclonal anti-iNOS ab3523 (Abcam) for chicken cells, rabbit anti-NOX2/gp91^{phox} polyclonal antibody conjugated with Alexa Flour 555 (Bioss), and mouse anti-*M. avium* monoclonal IgG1 BM1197 (Acris Antibodies. Inc. USA). The following secondary antibodies were used: goat anti rabbit IgG H&L (Alexa Flour® 555) (Abcam), goat anti rabbit IgG H&L (FITC) AB6717 (Abcam), goat anti-mouse IgG1 (FITC) sc-2078 (Santa Cruz Biotechnology, Inc. USA) and goat anti-mouse IgG1 gamma chain (Cy5®) preadsorbed ab136127 (Abcam).

2.18 Transmission Electron Microscopy (TEM)

For TEM, HD11 cells were seeded on coverslips and infected with *M. avium* strains Ma1 and Ma4 using the gentamicin protection assay as described before. At 6h and 24h post-infection, the infected cells

were washed three times with 0.1 M cacodylate buffer, and then the washed cells were fixed in 2% glutaraldehyde/ 0.1 M cacodylate for 1h at 4°C, then followed by washing twice with 0.1 M cacodylate buffer. The monolayers were post-fixed in 1% aqueous osmium tetroxide (OSO₄) for 30 min at room temperature, and then washed three times with distilled water before dehydration in a graded ethanol series starting from 50% and ending with 100%. The dehydrated cells were embedded in resin and kept at 60°C for 48h. Ultrathin sections were cut at 90nm and mounted on copper grids. The sections were stained with uranyl acetate and lead citrate and examined with a Tecnai G212 Bio Twin Digital TEM system. Photographs were taken at different areas of the grids.

2.19 Statistical analysis

Statistical analysis was done using GraphPad Prism 6 software. One-way ANOVA and *t*-tests were used for detection of significant differences among the experimental groups. Pearson's correlation tests were applied to evaluate correlations. Data were presented as mean concentration $\pm$ SE of three independent experiments. *p* values <0.05 were considered significant.

3.0 Interaction between *M. avium* strains and THP-1 and HD11 cells.

3.1 Introduction

Mycobacterium avium are intracellular bacteria which replicate and exist within the macrophages as chronic infections. Survival and proliferation of the bacteria inside phagocytic cells are considered to be important bacterial virulence factors (Rhoades and Ullrich, 2000). Phagocytosis of *M. avium* by macrophages is assumed to be an important step in the life cycle of the organism. The ingested mycobacteria inside macrophages exposed to an array of chemicals including reactive oxygen and nitrogen radicals (Yuan et al., 1995). Oxidative burst and nitric oxide have been suggested to play important roles in limiting the bacterial growth within macrophages (Bartos et al., 2004).

In addition to the initial entry of *M. tuberculosis* into host cells, it has been reported that the bacteria are able to damage the infected cells (Danelishvili et al., 2003, Gil et al., 2004). The destructive effect could be associated with bacterial virulence. It has been reported that both virulent H37Rv tubercle bacilli and *M. bovis* BCG invade the A549 human lung epithelial cells with only the pathogenic strain found to be cytotoxic for the human activated and non-activated epithelial cells and also macrophages (McDonough and Kress, 1995). Virulent strains of *M. tuberculosis* also produce

cytotoxic effects for human macrophages following four days of infection (Simeone et al., 2012).

However, most of studies on the interaction between *M. tuberculosis* and *M. bovis* and host cells were done on mammalian cells, and no single study, as yet, has been done on the interaction between *M. avium* and chicken cells. Therefore, the work reported in this chapter compares and describes in details the bacterial infection of chicken cells and the bacterial effect on the innate antimicrobial response in HD11 and THP-1 cells.

3.2 Results

3.2.1 Invasion and intracellular survival of *M. avium* strains in THP-1 and HD11 cell.

For invasion and intracellular survival of *M. avium* in THP-1 and HD11 cells the gentamicin protection assay was used. In this study, the sensitivity of the bacterial strains to gentamicin was evaluated. In the presence of gentamicin at a concentration of 100 ug/ml, all of the bacterial strains were killed and no single colony forming unit of the bacteria was observed on 7H10 agars even after 12 days of culturing of the bacterial suspensions. Besides, clear zone of inhibition around each disc of gentamicin was observed indicating that the bacteria strains unable to survive in the presence of the gentamicin. The results of invasion of mycobacterial strains in both cells lines are shown in Figure 3.1. The data showed that all strains were able to invade the cells used. However, there were differences between the levels of invasion. In THP-1 cells the viable numbers of invaded Ma5 were significantly greater than that of Ma1 ($p<0.01$) and Ma4 ($p<0.05$) and non-significantly higher ($P>0.05$) than that of Ma2 and Ma3. In contrast, in HD11, the numbers of viable bacteria recovered of Ma5 were much greater than Ma1, Ma3 ($p<0.01$) and Ma2 ($p<0.001$).

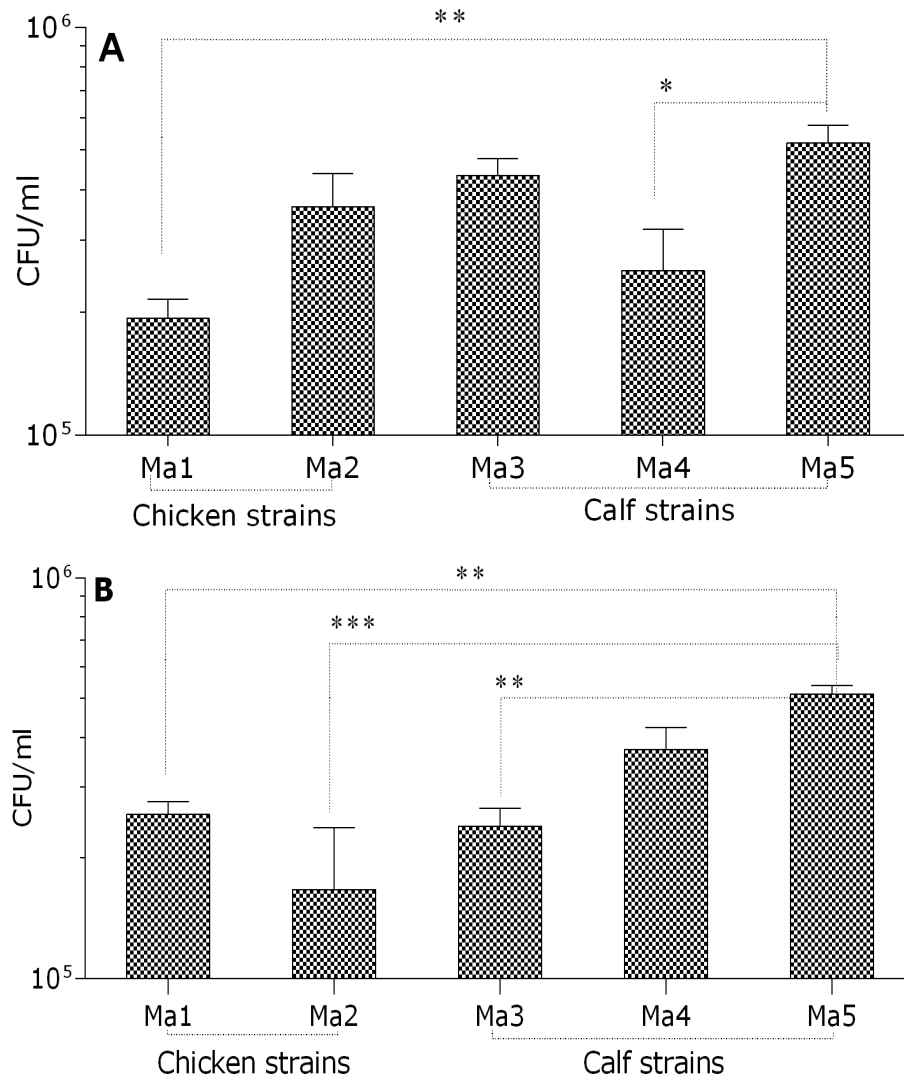


Figure 3.1: Invasion of the host cells with *M. avium* (A) THP-1 cells (B) HD11 cells. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627-08) and Ma5 (61-3728-08). Values shown as mean $\pm$ SE of six independent experiments with internal triplicate repeats. *p*-values indicate the significant difference between strain Ma5 and other strains. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

For further investigation on the invasion rates of the bacterial strains of HD11 cells, microscopic quantifications of infected HD11 cells with *M. avium* Ma1-Ma5 were used. The percentages of infected HD11 cells relative to non-infected cells were shown as (mean $\pm$ SE) of three independent experiments. The percentages of

infected cells were determined at 2h post-infection. Ma5 infected HD11 cells reported the highest percentage rates at (19%) and the lowest rate was reported by Ma2 at (7%). The results suggest that Ma5 was more efficient to invade the host cell than the other strains (Figure 3.2).

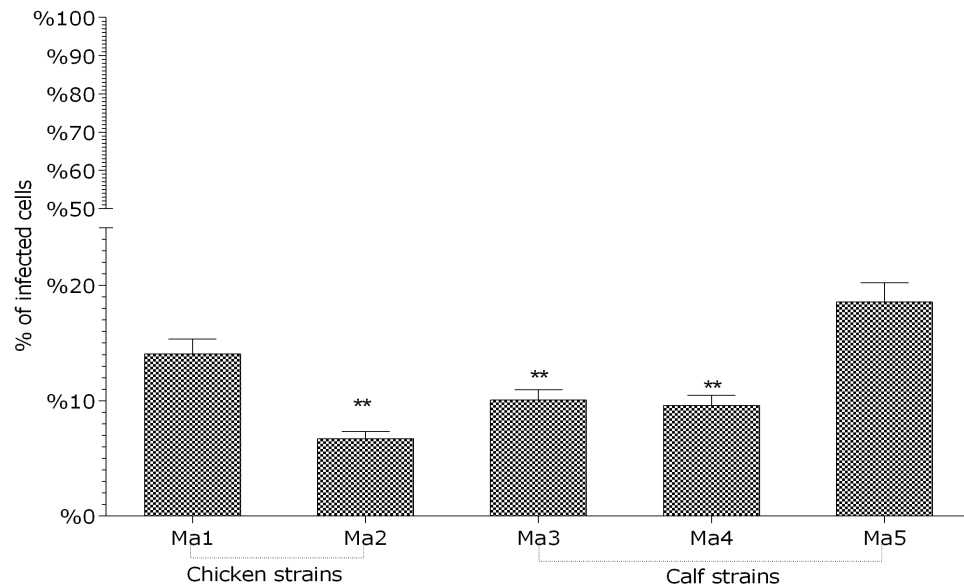


Figure 3.2: Percentage of HD11 cells infected at 2h post-infection. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08). The infected and non-infected cells were counted and infected rates were determined. Values shown as mean $\pm$ SE of three independent experiments performed in triplicates. ** indicates the statistically significant differences between the strain Ma5 and other strains. $p < 0.01$.

The intracellular kinetics of the bacterial strains were different between the cells lines used. In THP-1, the intracellular bacterial counts decreased at 6h post-infection followed by increasing by 24h (Figure 3.3 A). In contrast, in HD11, the number of viable bacteria of the bacterial strains began to increase at 6h and started to decline by 24h with the exception of Ma1 which increased during this time (Figure 3.3 B).

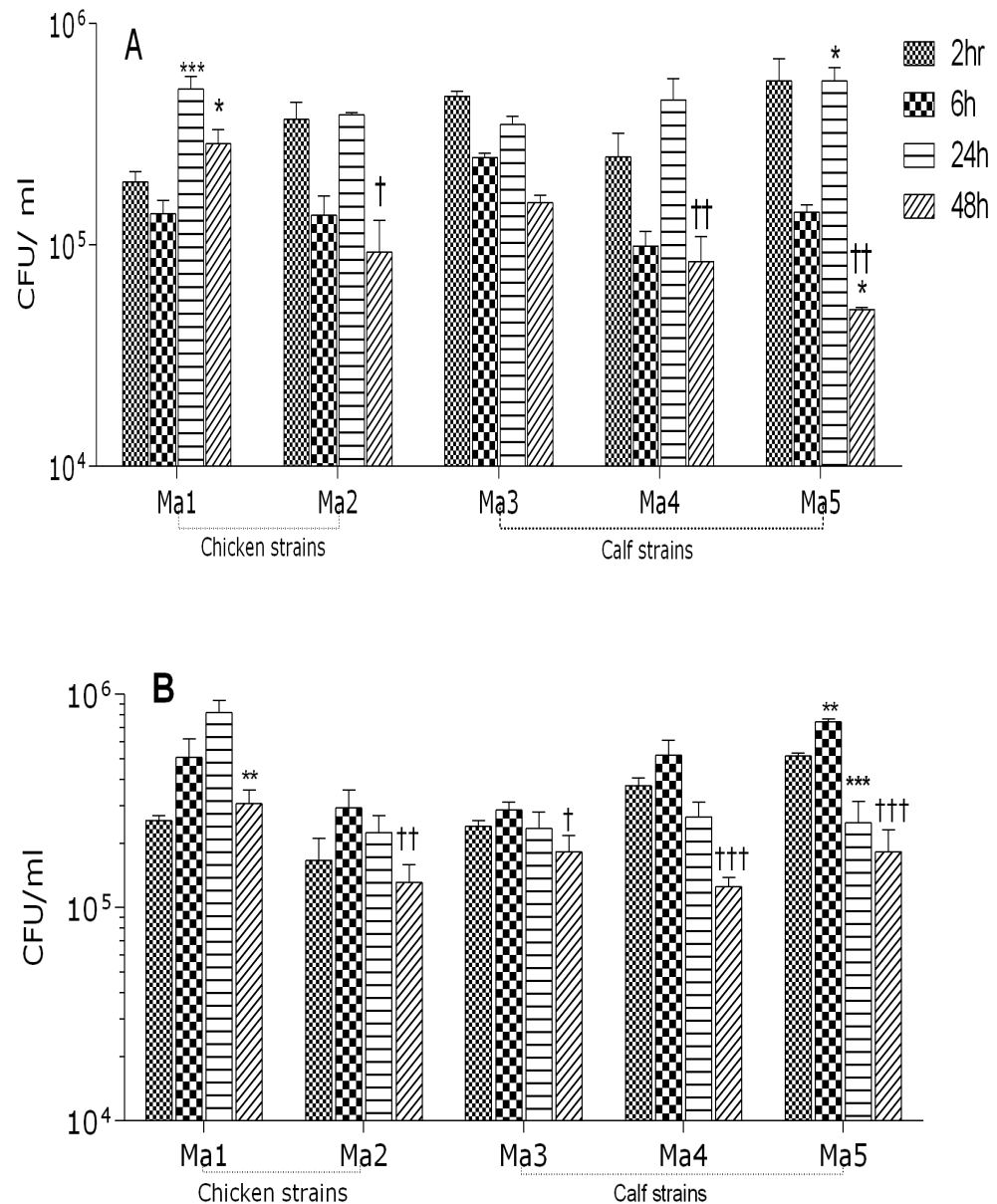


Figure 3.3: Intracellular survival and growth of *M. avium* in macrophages. (A) THP-1 cells (B) HD11 cells. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08). CFUs were determined at 2h, 6h, 24h and 48h point post-infection. Values shown as mean $\pm$ SE of six independent experiments each with triplicates infections. Significant differences in the CFUs changes of each strain between time points (2h and 6h, 6h and 24h, 24h and 48h) are indicated by asterisks. P -values * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$. (†) refers to significant differences $p < 0.05$ between CFUs of Ma1 and other strains at 48h PI.

The survival data showed that the viable numbers of Ma1 in THP-1 at 48h PI were significantly higher ($p < 0.05$) than Ma2 and highly significantly higher ($p < 0.01$) than that of Ma4 and Ma5, while Ma1 viable numbers were not significantly greater ($P > 0.05$) than Ma3 (Figure 3.3 A). In HD11 (Figure 3.3 B), the viable numbers of Ma1 at the same time point post-infection were significantly higher ($P < 0.01$) than that of Ma2 and Ma4 and higher than that of Ma3 and Ma5 at ($P < 0.05$). Thus, Ma1 and Ma3 are the most resistant strains to the intra-cellular hostile environment during 48h post-infection.

3.2.2 Intracellular counts:

The invasion and survival data in HD11 cells indicated an increase in bacterial numbers post-infection. The increases in the bacterial growth rates were Ma1, 33%; Ma2, 12%; Ma3, 16%; Ma4, 44% and Ma5, 28% by 6h after infection which was surprising. To determine if this was new growth or a result of bacterial fragmentation, we applied the FAM (Fluorescent Acid-Fast Microscopy) staining procedure and Transmission Electron Microscopy. Quantification of intracellular bacteria was carried out following a permeabilization of the membrane of the infected cells and staining the intracellular bacteria using the AO staining protocol. The intracellular bacterial counts gave nearly identical results to the colony forming units' cultures. The numbers of intracellular bacilli were counted by microscopy and culture. The data revealed that all five strains were able to replicate inside HD11. There were twofold increases in the

percentage of infected HD11 cells with two or more bacteria per cell between 2h and 6h post-infection, the rate of increase continuing during 24h and 48h post-infection to reach approximately three fold by 48h post-infection (Figure 3.4). The number of infected HD11 cells with single bacteria during 2h post-infection was greater than that with two or more bacteria which was nearly about two fold of HD11 with the single bacteria.

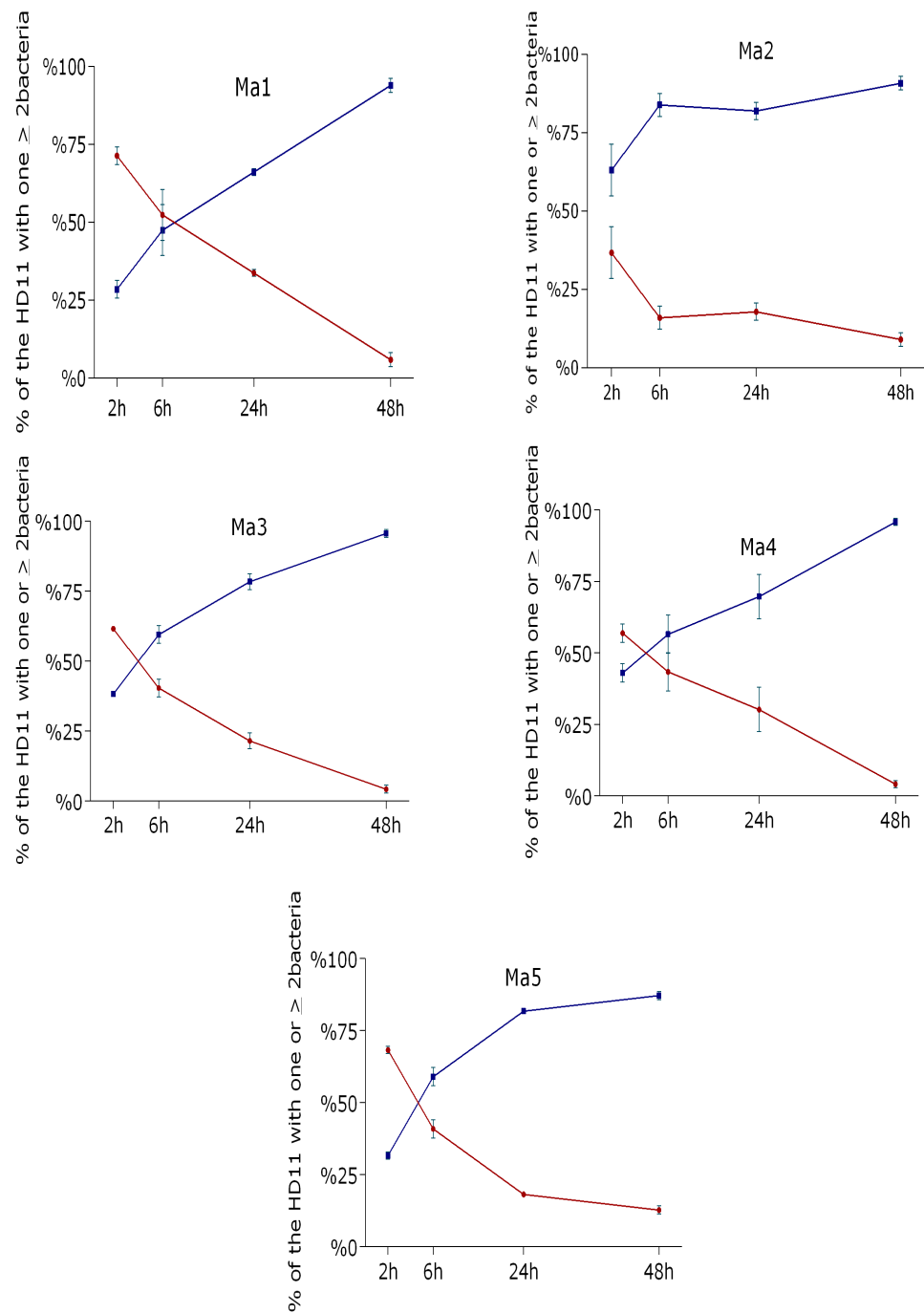


Figure 3.4: Percentage of HD11 cells containing single bacteria or two or more bacteria post-infection. Time points were 2h, 6h, 24h and 48h post-infection. Red line 1 bacterium per cell, blue line 2 or more bacteria. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559.07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08). Values shown as mean $\pm$ SE of three independent experiments with triplicates repeats for each sample per experiment.

To confirm that the increases within the number of infected cells with two or more bacteria per cell were not due to the transmission of the bacteria between the neighbouring cells, Transmission Electron Microscopy was used. All ingested bacteria were found within the membrane-bound compartment of the host cells, no bacteria were found free within cytoplasm of infected cells. At 6h post-infection, bacteria were found in a good condition, enlarged and some bacteria showed a clear elongation mode. A clear septal ring with two newly formed daughter cells also was found (Figure 3.5).

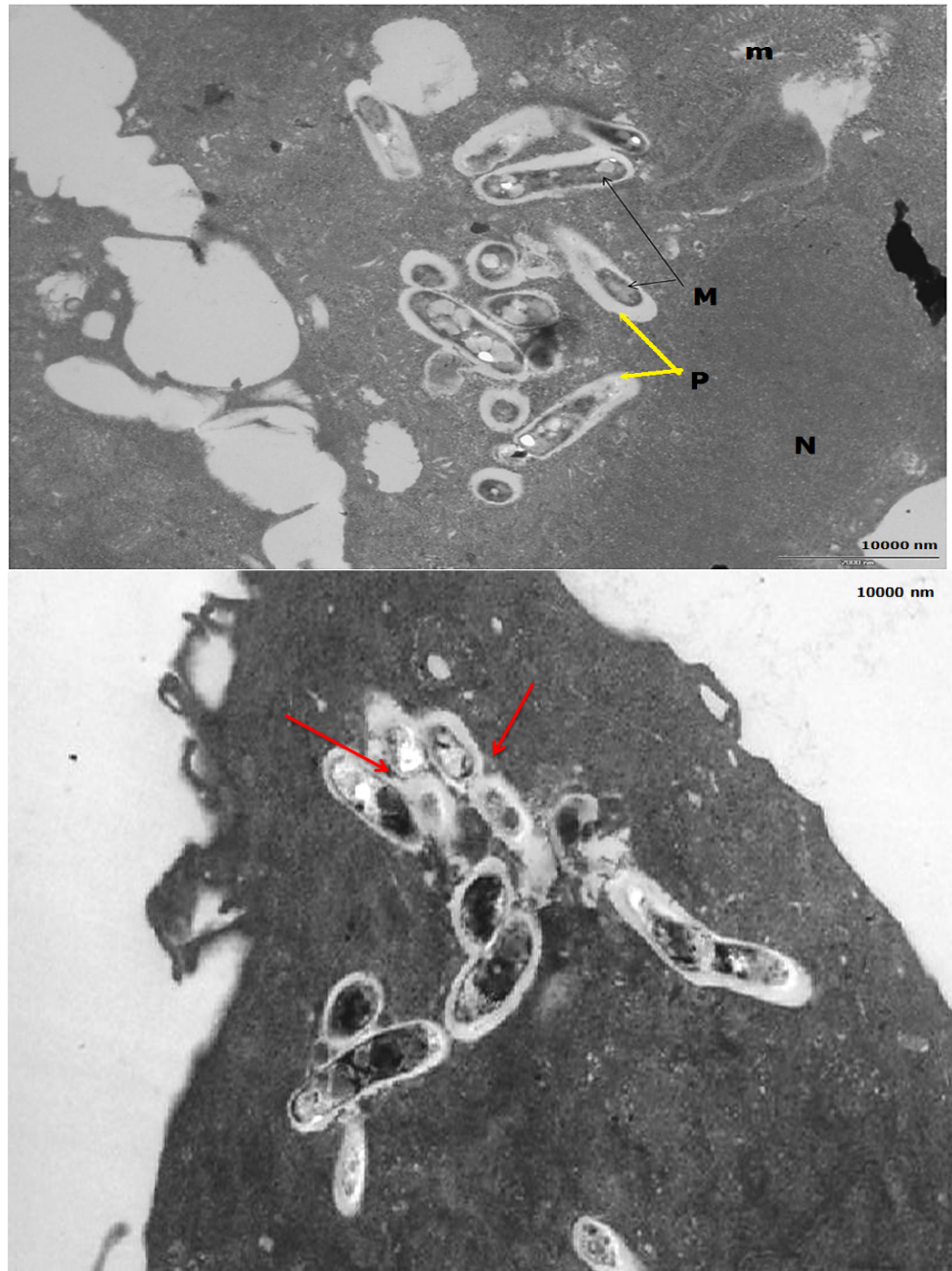


Figure 3.5: Representative TEM micrograph illustrating bacterial cell division in infected HD11 cells at 6h post-infection. Strain Ma4 (61-4627.08). Yellow arrows refer to phagosomes containing bacteria (single bacterium per a vacuole). Red arrows refer to bacterial cell division and the septal ring; bacterial increases in size. N=nucleus, P=phagosomes, M= *M. avium* and m= mitochondria.

By 24h post-infection bacteria were smaller than that were found at 6h after infection suggesting that the bacteria undergo a degradation and lysis (Figure 3.6).

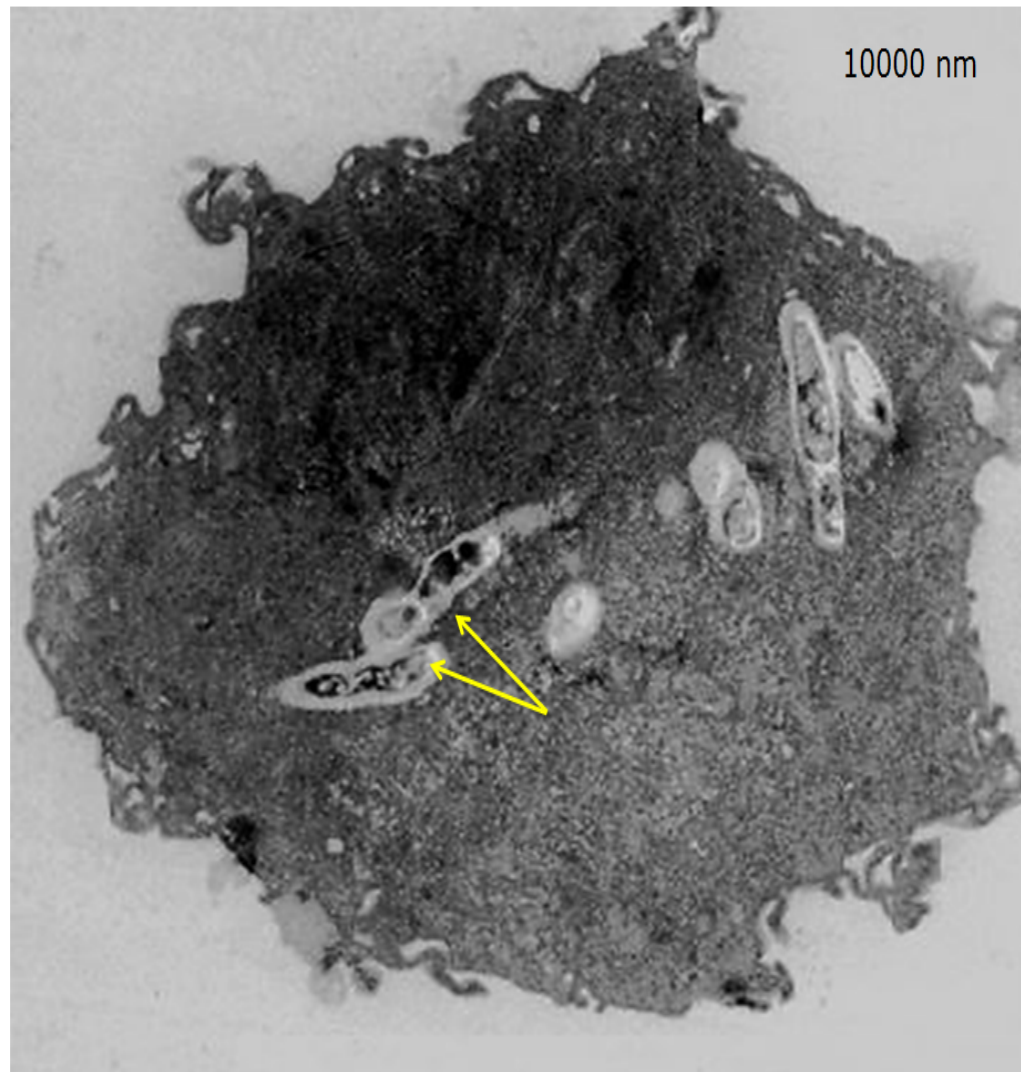


Figure 3.6: Representative TEM micrograph of infected HD11 at 24h post-infection. Strain Ma4 (61-4627.08). Yellow arrows refer to bacteria reduced in size showing shrunken and decomposed bacterial cell.

3.2. 3 Reactive oxygen species production by *M. avium*-infected THP-1 and HD11 cells

To determine the amount of reactive oxygen species (ROS) produced by *M. avium*-infected THP-1 and HD11, the nitroblue-

tetrazolium reduction assay was used. After infection, all strains of *M. avium* used were able to induce the cells to produce significant amounts of ROS above negative control (mock-infected cell) at all the time points post-infection (Figure 3.7). There were differences in the level of ROS production between HD11 and THP-1 cells and also between the bacterial strains tested. In infected THP-1 cells, the produced ROS levels were higher at 2h than that at the 6h post-infection. After 24h post-infection the produced levels of ROS by Ma1 and Ma2 were significantly increased ($p < 0.05$) and Ma3 and Ma4 ($p < 0.01$) above that at 6h post-infection. By 48h post-infection, ROS productions were reduced greatly. Equivalent strain to strain differences were also observed with HD11 infections. ROS production by the bacterial strains, with the exception of Ma1, was reduced at 6h compared with that of 2h post-infection; however, this was not statistically significant. The infected HD11 cells were able to produce high amount of ROS 24h post-infection in response to the bacterial infection and continued to increase ROS production by 48h post-infection which was in contrast to the finding in THP-1 cells.

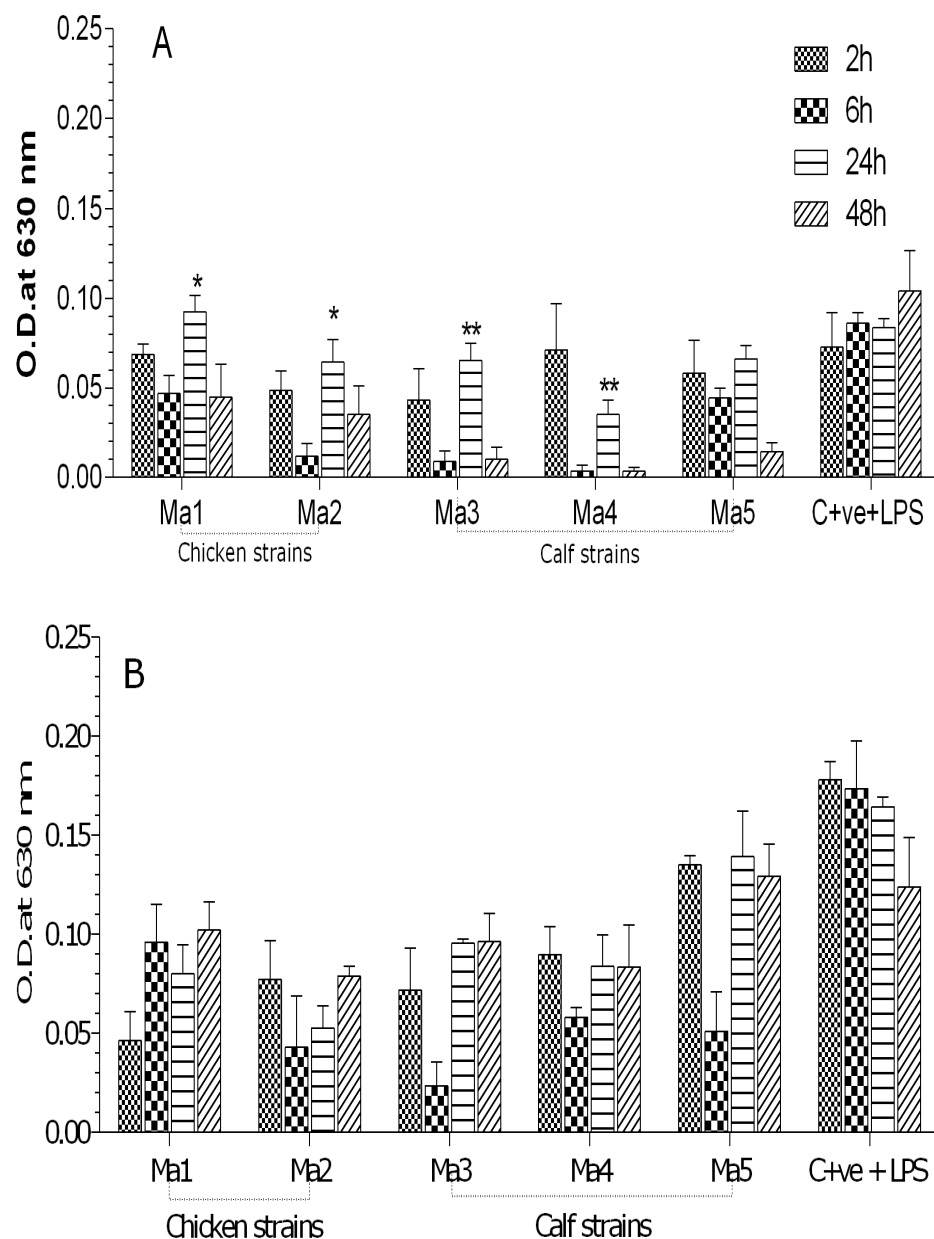


Figure 3.7: Induction of ROS production during *M. avium* infection. (A) THP-1 cells (B) HD11 cells. ROS level is expressed as increases of the optical density at 630 nm above negative control of mock-infected challenged with PBS. Positive controls of HD11 and THP-1 cells were incubated with PMA at 100 ng/ml and challenged with 10ul of LPS at 50 µg/ml as a final concentration. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08). Values shown as mean $\pm$ SE of three independent experiments with internal triplicate repeats. Significant differences in ROS changes of each strain between time points are indicated by asterisks * = $p < 0.05$, ** = $p < 0.01$.

3.2.4 Nitric oxide production by *M. avium* infected cells

Experimental infection of HD11 with different strains of *M. avium* induced different but increasing concentrations of nitrite at the time points post-infection. There were no significant differences between mycobacterial infected HD11 and non-treated cells (control negative) in nitric oxide produced during 2h and 6h post-infection. The maximum nitric oxide production was found by 48h post-infection (Figure 3.8). Nitric oxide production by Ma4 and Ma5-infected cells at 48h post-infection was significantly higher ($p < 0.001$) than that produced by Ma1, Ma2 and Ma3 infected cells. As expected THP-1 infected cells did not produce nitric oxide at any time post-infection.

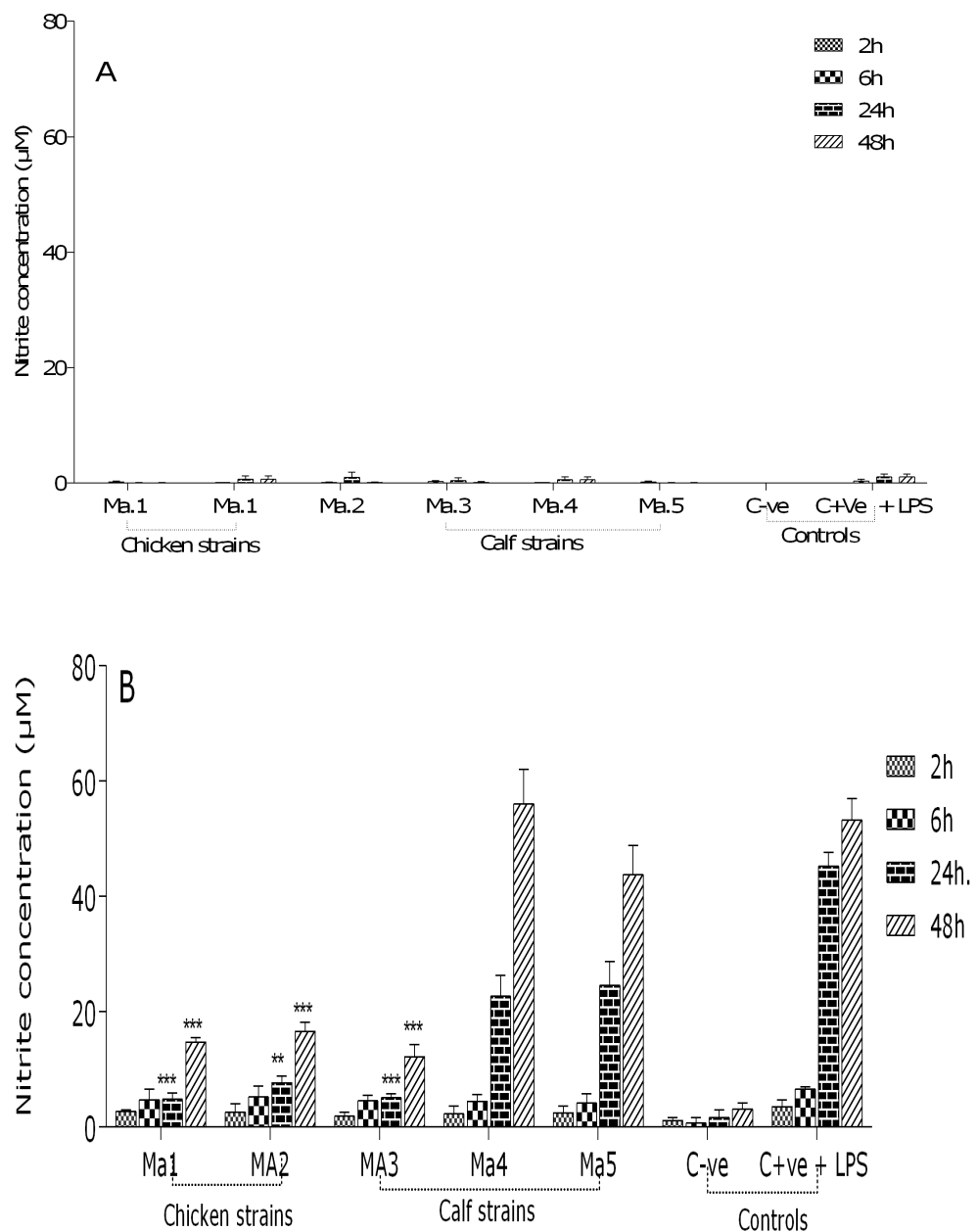


Figure 3.8: Nitric oxide production by *M. avium* infected cells at different time point post-infection. (A) THP-1 cells (B) HD11 cells. Negative control of mock-infected challenged with PBS. Positive controls of HD11 and THP-1 cells were challenged with 50ul of LPS at 50 $\mu\text{g}/\text{ml}$. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08). Values shown as mean $\pm$ SE of three independent experiments with internal triplicates repeats. Significant differences in the levels of nitric oxide produced by Ma4 and other strains at each time points are indicated by asterisks ** = $p < 0.01$ and *** = $p < 0.001$.

3.2.5 Cytotoxicity assay

The release of lactate dehydrogenase (LDH) was used to detect cytotoxic effects during infection of the cells. LDH was measured at different time points post-infection and compared with LDH released from non-infected cells (negative controls). In THP-1, no cytotoxic effects were observed over a period of 48h, while, in HD11, the cytotoxic effects were evident at 2h after infection by all of the bacterial strains except Ma2 and the highest LDH was 12.5% released by Ma3 infected HD11 cells (Figure 3.9). Interestingly, the data showed that the cytotoxicity was negligible or significantly reduced ($p < 0.01$) at 6h and 24h post-infection compared to the produced levels of LDH at 2h post-infection. At 48h post-infection, the LDH released by the infected HD11 cells was greatly increased compared with the values at 24h post-infection.

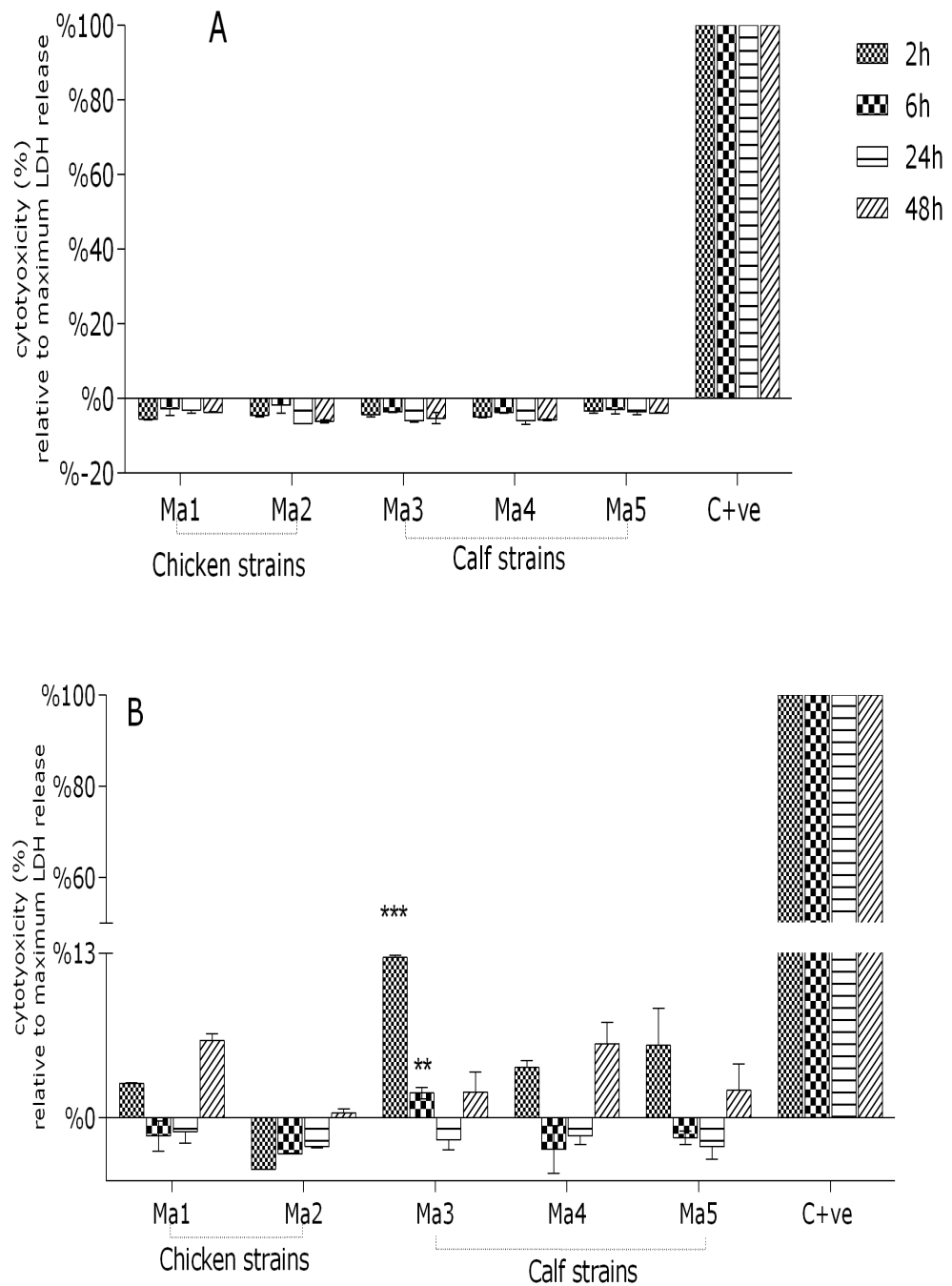


Figure 3.9: Measurement of cytotoxic effects of *M. avium* on cells by LDH assay. (A) THP-1 cells. (B) HD11 cells. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08). Values shown as mean $\pm$ SE of three independent experiments performed with internal triplicate repeats. Significant differences in the levels of released LDH by infected cells with each strain between time points are indicated by asterisks. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

3.6 Discussion

In order to better understand the interactions between *M. avium* subspecies *avium* and the human and avian host cells at the cellular level, the invasion and survival of *M. avium* strains were investigated in human macrophage-like cell lines, THP-1 (human) and HD11 (chicken). THP-1 and HD11 cells were infected with five different strains of *M. avium*, two poultry isolates and three bovine isolates. All of the bacterial strains tested were able to invade and replicate within the host cells. There were strain to strain differences in the initial invasion levels. The highest invasion rates in both THP-1 and HD11 cells were reported for Ma5, calf isolate. However, the mechanisms by which *M. avium* invade the host cells are not fully understood, the variations between the strains are probably due to the ability of the bacteria to utilize different receptors to invade the host cells; further discussions on the bacterial invasion and the role of phagocytic receptors can be seen within the next chapter, chapter four. It has been seen that the virulent strains of mycobacteria, *M. tuberculosis* and *M. celatum* were more efficient in invading THP-1 than that of avirulent strains of mycobacteria, *M. abscessus* and *M. fortuitum* (Helguera-Repetto et al., 2014). The bacteria may utilize different receptors to interact with the phagocytic cells, and / or there may have been variation in the bacterial ability to interact with the host cell cytoskeleton to enter into the host cells. Ernst (1998) stated that *M. tuberculosis* entered human macrophages via a

variety of receptors. Bermudez and Goodman (1996) found that *M. tuberculosis* strain H37Rv entered alveolar epithelial cells A549 more efficiently than H37Ra, indicating a differences between strains of the bacteria in invading the host cells.

The present study showed that the infected macrophages were unable to completely remove any of the bacterial strains over 48h post-infection. However longer term persistence of bacteria would be hard to assess in cell culture due to the cells viability drop off after this time. During the 48h period all the bacterial strains could persist within the cells however the kinetics of the intracellular survival of the bacterial strains were different between human (THP-1) and the avian (HD11) cells. The viable numbers of the bacterial strains had decreased in THP-1 by 6h post-infection and were followed by increases in their numbers by 24h post-infection. Similar results were reported for *M. abscessus*-infected THP-1 at 6h and 24h, whereas, for pathogenic *M. tuberculosis* there were no differences between the viable numbers over different time point post-infection, 6h, 24h and 48h post-infection (Helguera-Repetto et al., 2014). In contrast to THP-1, in HD11, the numbers of the bacteria began to increase at 6h and started to decline during the periods of 24h and 48h except for Ma1 which increased by 24h post-infection. The differences in the bacterial numbers between two cells at 6h and 24h might be due to the effect of ROS and nitric oxide. The infected cells, THP-1 and HD11 cells were able to produce ROS

in response to *M. avium* Ma1-Ma5 infections, whereas, nitric oxide was only produced by infected chicken cells in response to intracellular organisms. In context of nitric oxide production, the present data are in agreement with Setta et al. (2012) who reported that HD11 cells produced significant amount of nitric oxide in response to *Salmonella* Enteritidis infection. Roshick et al. (2006) reported that THP-1 cells do not produce nitric oxide under any condition even in when co-treated with IFN- γ and LPS.

The increased numbers of the bacterial strains within the infected cells were associated with increased level of ROS production, particularly at 24h post-infection in infected THP-1 cells. However, it is greatly accepted that ROS restricts the growth of intracellular pathogens such as *Salmonella* Typhimurium in murine macrophages (Lajarin et al., 1999, Mantena et al., 2008) and *Campylobacter jejuni* in murine and porcine macrophages (Day et al., 2000), here we have found that the intracellular kinetic of *M. avium* is associated with the ROS production suggesting that ROS plays an important role in the intracellular survival of the bacteria in infected THP-1 and HD11 cells. These findings are in accordance with those reported by Meylan et al. (1992) who found that the intracellular survival of *M. tuberculosis* and *M. avium* in human macrophages were enhanced under the presence of high concentration of oxygen. In contrast to pathogenic mycobacteria, non-pathogenic mycobacteria, such as *M. abscessus* and *M.*

fortuitum, have been reported to induce THP-1 to produce more ROS, as a result the bacterial numbers were greatly reduced by 24h post-infection (Helguera-Repetto et al., 2014) The roles of ROS in intracellular survival of *M. avium* in infected THP-1 and HD11 cells are further investigated in chapter five .

Although infected HD11 were able to produce higher amounts of ROS than that of THP-1, the numbers of the bacterial strains were reduced by 24h and 48h post-infection, where, the nitric oxide production by infected cell was significantly increased, particularly by Ma4 and Ma5, compared to the levels that were produced by Ma1 and Ma3 infected HD11. In addition, the numbers of Ma1 and Ma3 recovered from infected HD11 cells at 48h post-infection were significantly higher than that of the other strains, suggesting that nitric oxide could have important roles in suppressing the intracellular survival of *M. avium* within infected HD11, but not in THP-1 cells. The resistance of *M. tuberculosis* to induced nitric oxide depends on strain, amount and time of exposure. It has been found that pathogenic mycobacteria are more resistant to nitric oxide, compared to non-pathogenic strains (Long et al., 1999). Macrophages exert their antimicrobial activity through nitric oxide and oxidative burst (Appelberg, 2006). The present study found that the infected HD11 cells produced different concentration of nitric oxide in response to *M. avium* strains infections; induced nitric oxide played a role in the bacterial survival within infected cells

particularly at 24h and 48h post-infection. Further studies on the role of nitric oxide in intracellular survival of the mycobacteria within the infected HD11 cells were done using nitric oxide stimulators and inhibitors; data can be seen in chapter five.

The observed reduction in the number of intracellular bacteria of *M. avium* at 48h in human (THP-1) and avian (HD11) cells might be due to the cytotoxicity of the bacterial strains. Lactate dehydrogenase (LDH is a cytoplasmic enzyme which is released as a result of damage of plasma membrane and cell death) was measured at different times point post-infection (2h, 6h, 24h and 48h). However, we found that all of the bacterial strains had some cytotoxic activity on the HD11 cells compared to THP-1 where there was no cytotoxicity. In HD11, there were strain to strain differences in the level of LDH induction at 2h post-infection and the maximal LDH was released by Ma3 12.5% compared to the other strains. The early release of LDH at 2h post-infection is probably due to the use of a relatively high MOI 10:1 (10 bacteria per HD11 cells), high invasion rates and the bacterial pathogenicity. These findings are in line with those reported by McDonough and Kress (1995) who found that the cell death of human pneumocytes was enhanced by invasion with virulent H37Rv tubercle bacilli compared to attenuated *M. bovis* BCG vaccine strain. In contrast to the maximal LDH released by lysis of uninfected cells, LDH released by *M. avium*-infected HD11 at 6h and 24h post-infection was not elevated above that released by the non-infected cells. This might be as invasion

has already occurred and is little after 2h, the limited lysis observed related to the bacterial invasion process. The level of LDH released by infected HD11 at 48h post-infection was significantly increased compared to that released by 24h, but in fact the levels produced were not as expected especially when it was shown microscopically that the number of infected HD11 were greatly reduced at 48h post-infection compared to non-infected cells. The increase in LDH release at 48h post-infection is probably due to the intracellular growth of the bacteria leading to cell death; among the infected cells, the number of HD11 cells containing two or more bacteria increased to over than 90% at 48h post-infection. Thus, the cytotoxicity data suggested that the bacterial cytotoxicity may not play a significant role in intracellular survival of the bacteria.

In summary, this study provides data on invasion and survival of *M. avium* in human monocyte-derived macrophages and chicken cells. All of the bacterial strains were able to invade and survive within the cell lines used. Survival data showed differences between human and chicken cells in intracellular survival kinetics of *M. avium*. Data showed a rapid division of the bacterial cells at 6h post-infection in HD11 cells, while the viable numbers of the bacteria were reduced at the same time in THP-1 cells. Both THP-1 and HD11 produced ROS in response to mycobacterial infection, whereas, only HD11 cells were able to produce nitric oxide. However, cytotoxicity data indicated that all strains were more cytotoxic to HD11 in comparison with THP-1 cells, but the cytotoxic effect of the bacterial strains did

not appear to be significant for the survival kinetics of the bacteria strains.

4.0 Mechanisms of invasion of the host cells by *M. avium*.

4.1 Introduction

The interaction of mycobacteria with macrophages is central to the development of disease in both avian and mammalian hosts (Dietrich and Doherty, 2009, Ignatov et al., 2012). We observed differences between our test strains of *M. avium* in invasion and were interested in determining if these related to differences in invasion mechanisms. In mammals *M. tuberculosis* have been shown to use a range of different strategies to invade the host macrophages (Ernst, 1998). Initial invasion can proceed via three receptors, complement receptors (CR), mannose receptors and scavenger receptors (Hossain and Norazmi, 2013, Caron and Hall, 1998, Astarie-Dequeker et al., 1999, Schlesinger, 1993, Schäfer et al., 2009, Zimmerli et al., 1996).

Serum factors have also been shown to contribute to the invasion of macrophages by *M. tuberculosis*, *M. leprae*, and *M. avium* (Schorey et al., 1997) and mycobacteria have been shown to activate the complement system through different pathways leading to cleavage of C3 to produce C3b which is essential for CR3 mediated entry into human macrophages (Ferguson et al., 2004, Van Crevel et al., 2002). However mycobacteria can also invade mammalian cells in the absence of sera using mannose receptors (Schlesinger et al.,

1996, Zimmerli et al., 1996). In the absence of mannose receptor and the CR3 receptor invasion can also be mediated by scavenger receptors (Zimmerli et al., 1996). The reported results therefore indicate that there are a range of receptors which can mediate invasion.

Actin and tubulin have been reported to play important roles in the early phase of the interaction between mycobacteria and the mammalian phagocytes to generate the forces required for the actual phagocytosis (Ferrari et al., 1999). Remodelling of actin and tubulin is central to the invasion but their roles in relation to the different receptor mediated mycobacterial invasion is unclear (Ferrari et al., 1999, García-Pérez et al., 2003, Casali et al., 2002).

In comparison with studies on mammalian infections with *M. tuberculosis* and *M. bovis* no studies have described the invasion receptors for *M. avium* with avian cells. As the route of entry to cells can lead to different compartmentalisation this can have a major impact on the bacterial survival and defining these is critical to understanding how the bacteria persist within the infected cells.

The work reported in this chapter compares and describes the receptors and mechanisms required for invasion by *M. avium* in both avian and human cells.

4.2 Results

4.2.1 The role of different sera in *M. avium* invasion into the host cells

The role of different sera on opsonisation and phagocytosis of the *M. avium* strains by THP-1 and HD11 cells was examined. Infections were carried out with either human, foetal calf or avian serum and the impact on invasion monitored. As shown in figure 4.1A, phagocytosis of all 5 bacterial strains by THP-1 cells was significantly enhanced ($p < 0.01$) in the presence of human serum (HS) compared to serum free RPMI. It was also higher than the values produced by using sera from chicken or calf. In contrast to THP-1, bacterial invasion of HD11 was only significantly enhanced by chicken serum for strains Ma1 and Ma5 whereas uptake of all strains except Ma3 was enhanced by foetal calf serum ($p < 0.05$) (FCS, Figure 4.1B). Phagocytosis of bacteria by HD11 cells decreased in the presence of the human serum but this was not statistically significant ($p > 0.05$).

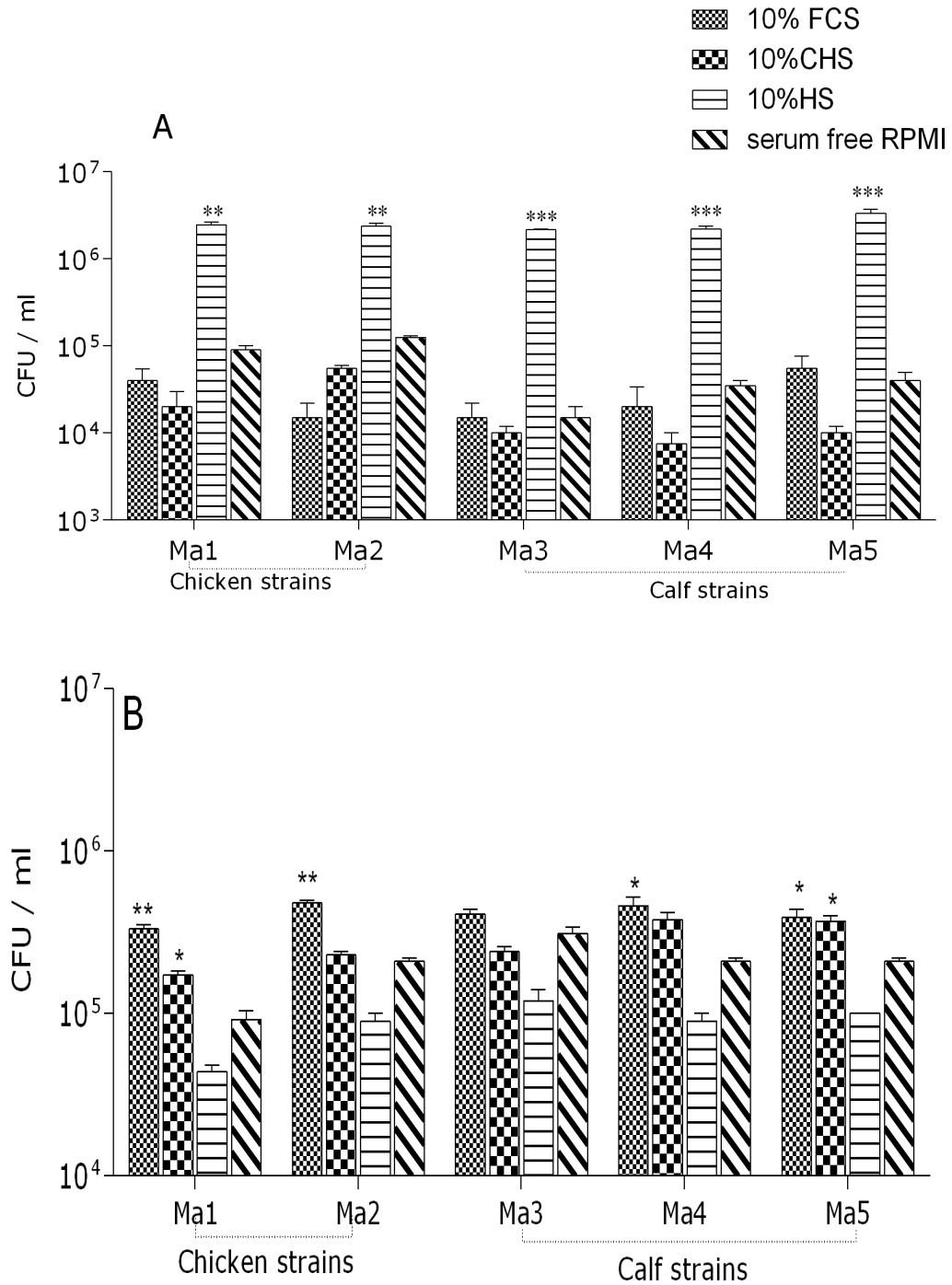
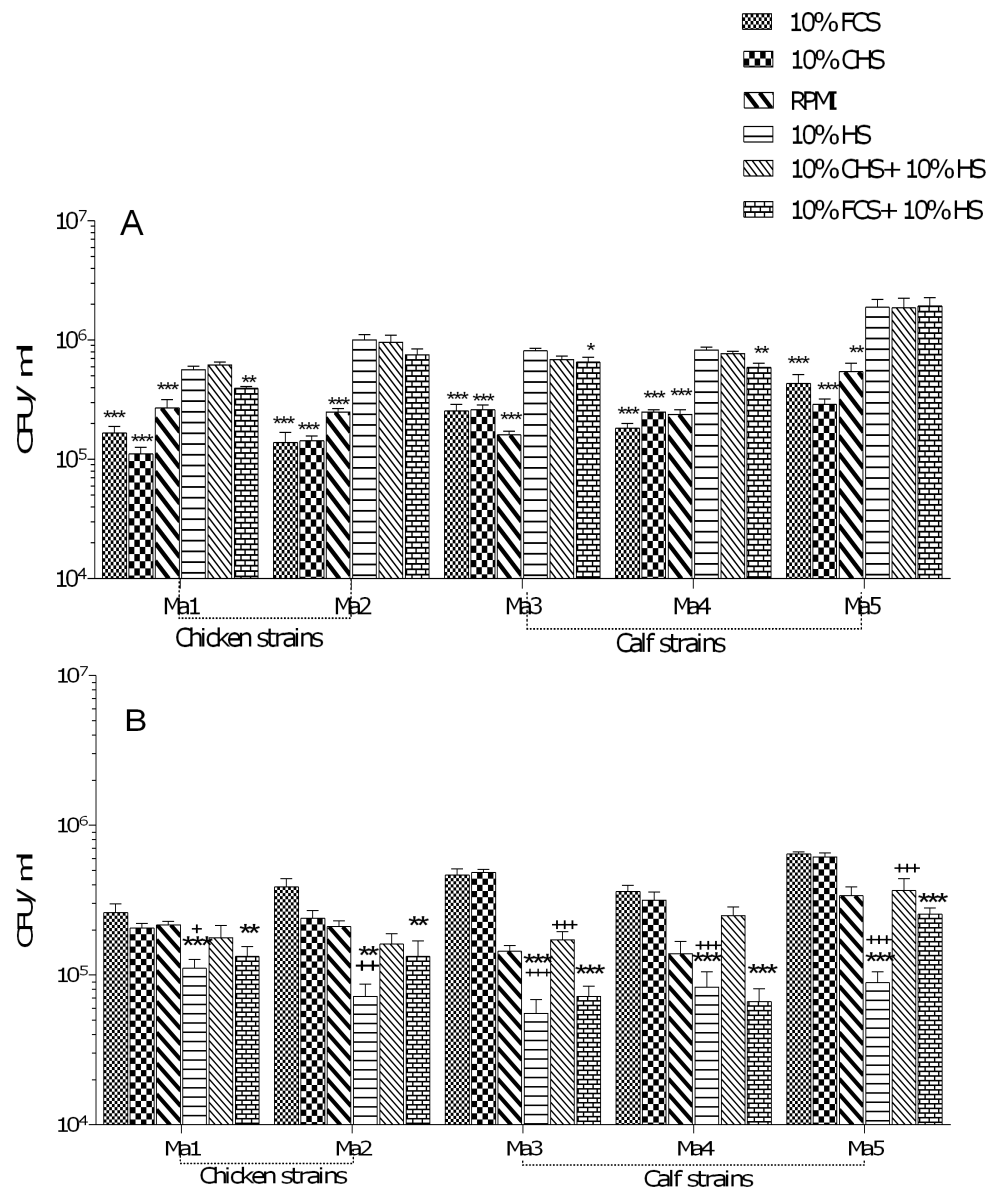


Figure 4.1: Role of different sera on *M. avium* invasion of macrophage cells (A) THP-1 cells. (B) HD11 cells. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08). FCS-foetal calf serum, CHS-chicken serum and HS-human serum. All sera were added at a final concentration of 10% v/v. Control infection was serum free RPMI. The values shown are the means $\pm$ SE for three separate experiments with internal triplicate repeats. Significant differences * $p < 0.05$ and ** $p < 0.01$ and *** $p < 0.001$ between serum free RPMI and serum groups.

To further understand the difference in impact of different host sera and their possible inhibitory effects on bacterial phagocytosis by the host cells, combinations of different host sera were compared for inhibitory properties. HS+CHS and HS+FCS were used in combination each at a concentration of 10% v/v in the invasion assays. The data found that chicken sera had no significant inhibitory effect on *M. avium* Ma1-Ma5 phagocytosis by THP-1 in the presence of HS. In contrast to CHS, FCS had a significant ($p < 0.05$) effect on Ma1, Ma3 and Ma4 phagocytosis in the presence of human sera and non-significant ($P > 0.05$) inhibitory effect on Ma2 and Ma5 (Figure 4.2A). In contrast human sera was found to have a statistically significant inhibitory effect on the *M. avium* Ma1-Ma5 phagocytosis when it mixed with FCS ($p < 0.01$) (Figure 4.2B).



Initial studies were carried out with serum which had been stored frozen at -20°C. Frozen storage of serum can have an impact on opsonisation proteins. To account for this potential impact invasion was compared using both fresh and frozen sera. Invasion of the *M. avium* strains into THP-1 and HD11 cells was significantly increased in the presence of fresh human and chicken sera compared to the frozen sera ($p < 0.05$). However no significant differences were reported within the number of viable bacteria invaded into HD11 in the presence of fresh compared to frozen FCS (Figure 4.3A and B).

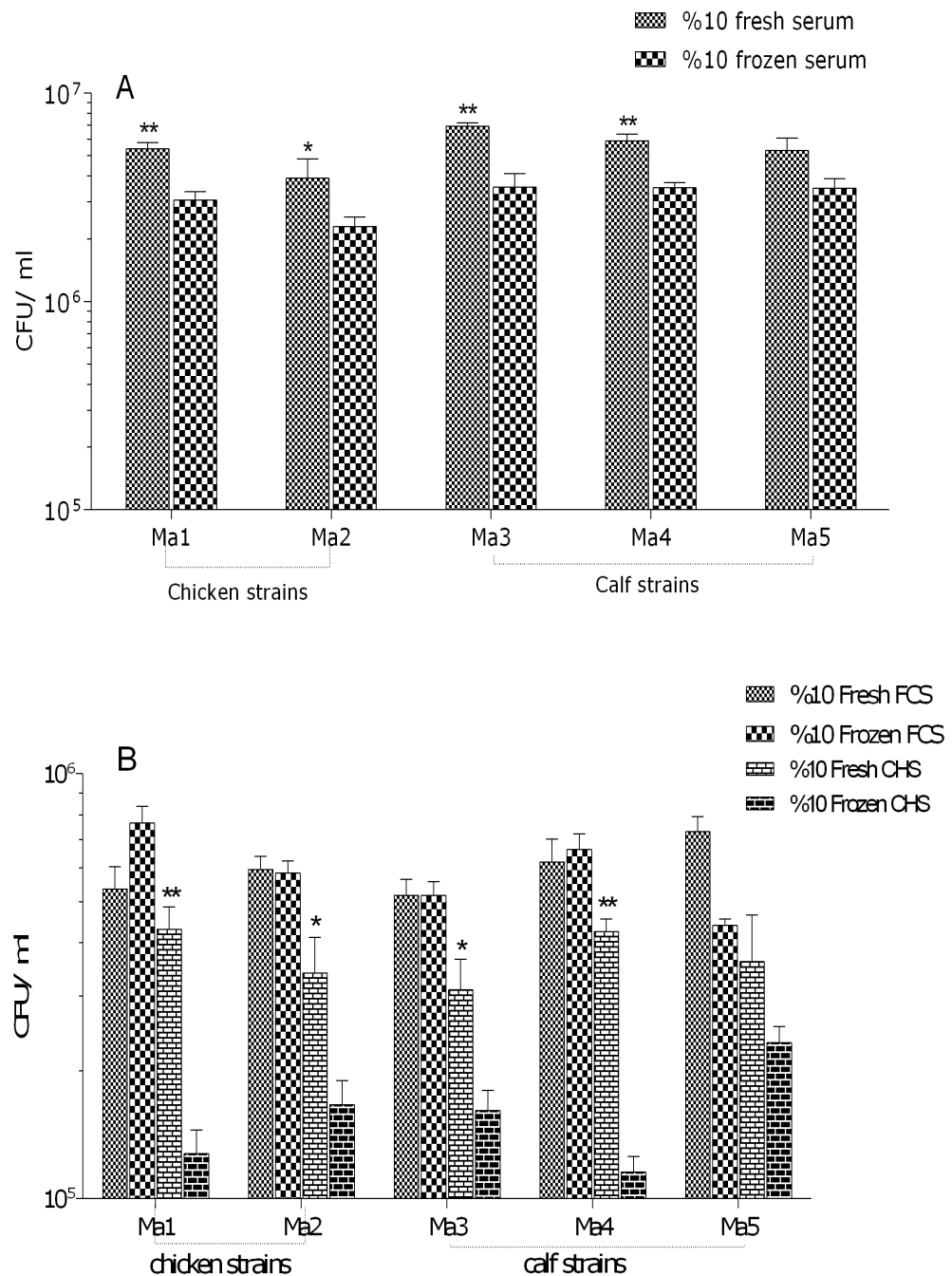


Figure 4.3: Difference between invasion level of macrophage with fresh and frozen serum. (A) Invasion of THP-1 human cells with human serum **(B)** Invasion of HD11 with foetal calf and chicken serum. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627-08) and Ma5 (61-3728-08). FCS-foetal calf serum, CHS-chicken serum and HS-human serum. The values shown are the means $\pm$ SE for three separate experiments with internal triplicate repeats. Significant differences * $p < 0.05$ and ** $p < 0.01$ between fresh and frozen serum of the same species.

4.2.2 Complement system and *M. avium* invasion into the host cells.

To investigate the activation pathways that *M. avium* uses to activate the complement system, EGTA and EDTA were used to distinguish the dominant complement pathway during infection of the cells. EGTA and EDTA differentially inactivate the classical and alternative complement respectively. As shown in Figure 4.4 the phagocytosis of all five *M. avium* strains in THP-1 and HD11 were reduced in the presence of either EGTA and EDTA and with heat inactivated serum ($p < 0.001$). These results suggest that the classical pathway is a predominant pathway for complement activation during bacterial invasion (figure 4.4, 4.5).

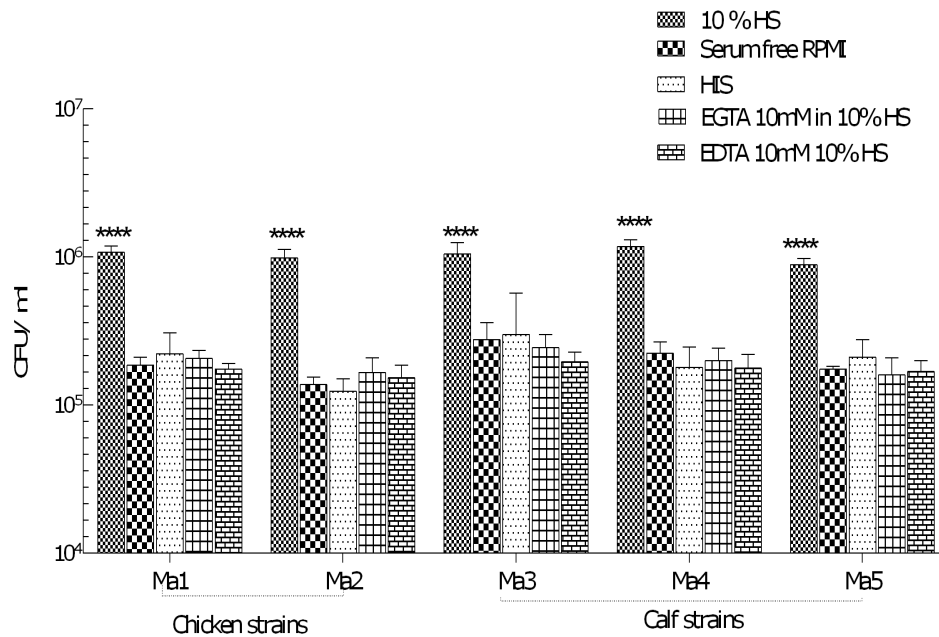


Figure 4.4: The role of human serum complement in opsonisation and subsequent invasion of *M. avium* of THP-1 cells. Positive control 10% human serum (HS). Negative control serum free and no chelators. Treatments, EGTA treatment 10 mM with human serum at 10%, EDTA treatment 10 mM with human serum at 10% and heat inactivated serum with no chelators (HIS). Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08). The values shown are the means $\pm$ SE for three separate experiments with internal triplicate repeats. Significant differences **** $p < 0.001$ between non-treated 10% v/v human serum and other treated serum groups and serum-free RPMI reported by each mycobacterial strain.

The effect of complement components in animal sera (FCS and CHS) on invasion of HD11 cells was also investigated using chelators and heat inactivated serum. The infections were carried out in the presence or absence of treated FCS and CHS and compared with the non-treated sera. As was observed for THP-1 cells significant reductions were observed for both heat-inactivated and chelator-treated samples (Figure 4.5, 4.6).

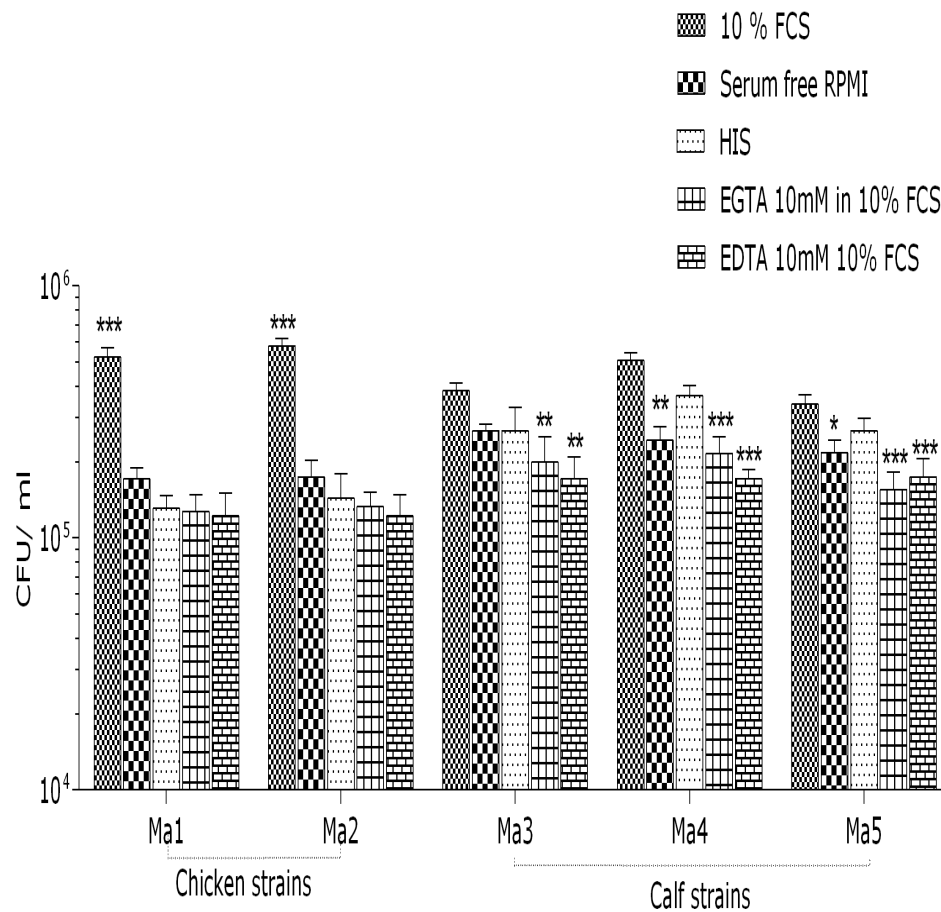


Figure 4.5: Roles of FCS complement in opsonisation and subsequent invasion of *M. avium* of HD11 cells. Positive control 10% foetal calf serum (FCS). Negative control serum free and no chelators. Treatments, EGTA treatment 10 mM with FCS at 10%, EDTA treatment 10 mM with FCS at 10% and heat inactivated serum with no chelators (HIS). Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08). The values shown are the means $\pm$ SE for three separate experiments with internal triplicate repeats. Significant differences * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ between non-treated 10% v/v FCS and other treated groups and serum-free RPMI reported by each mycobacterial strain.

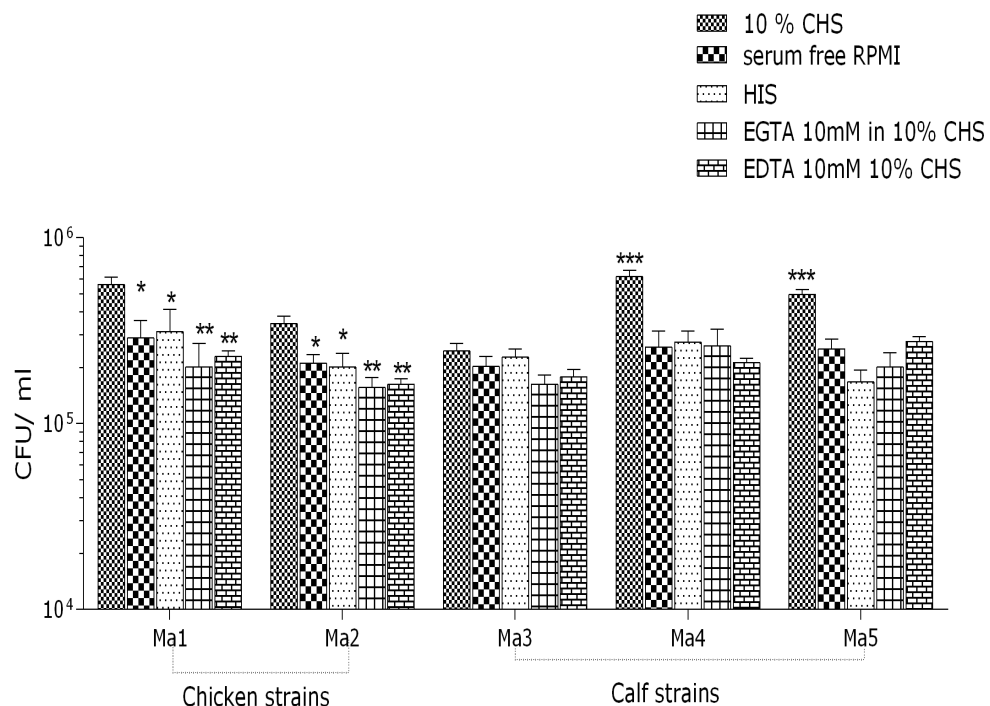


Figure 4.6: Roles of chicken serum complement in opsonisation and subsequent invasion of *M. avium* of HD11 cells. Positive control 10% chicken serum (CHS). Negative control serum free and no chelators. Treatments, EGTA treatment 10 mM with CHS at 10%, EDTA treatment 10 mM with CHS at 10% and heat inactivated serum with no chelators (HIS). Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08). The values shown are the means $\pm$ SE for three separate experiments with internal triplicate repeats. Significant differences * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ between non-treated 10% v/v CHS and other treated groups and serum-free RPMI.

4.2.3 The role of mannose-, scavenger- and complement receptors in the bacterial invasion into the host cells.

While EGTA and EDTA reduced invasion there was still a significant level of invasion suggesting that other mechanisms of invasion were also involved in uptake of the bacteria. To determine the role of mannose-, scavenger- and complement receptors during invasion experiments were carried out using the competitive chemical

ligands. α -methyl *D*-mannoside (2%), dextran sulphate sodium salt (100 ug/ml) and heparin sodium from porcine intestinal mucosa (10 U/ml) respectively. These were assessed both independently and in combination. The mannose and scavenger receptors studies were carried out in serum-free infection, while the importance of CR3-mediated bacterial invasion was determined in the presence of 10% v/v of host sera specific to the species of cells used. The data are presented as the percentages of the bacterial invasion relative to negative controls which had no inhibitor present.

Independent blocking of all the receptors led to a reduction of invasion in both human and avian cells. The reduction of invasion by different inhibitors was bacterial strain dependent. In THP-1 cells inhibition of mannose receptor binding was most consistent between strains with a reduction in invasion of 60% – 80%. There were clear strain dependent differences for both scavenger receptor and complement CR3 mediated invasion across strains (Figure 4.7 A).

In contrast mannose receptor blocking was less effective in inhibition of invasion with HD11 cells compared with THP-1 cells with a reduction of 40% - 50% compared to the negative control. As was observed in assays with human cells there was more variation between strains for blocking with scavenger- and complement CR3 receptors (Figure 4.7 B). There were variations between bacterial strains and host cell types observed for the contribution of the different receptors in invasion by *M. avium*.

In THP-1 cells that were simultaneously treated with all three receptor inhibitors the bacterial invasion was reduced compared to cells that were treated with individual blocker ($p < 0.01$), except Ma1 in mannose receptors and scavenger receptors and Ma2 in mannose receptors blocked cells ($p > 0.05$) (Figure 4.7 A). The simultaneous blocking of receptors inhibited bacterial invasion by *ca.* 65% for Ma1, Ma2 and *ca.* 85% for Ma3, Ma4 and Ma5. In HD11 cells, the simultaneous blocking of the receptors did not inhibit the bacterial phagocytosis further than that of scavenger receptor blocked cells, except Ma4. Thus, the data suggest that the phagocytic receptors are not working independently but they cooperate to interact with different ligands on the mycobacterial surface simultaneously for optimal binding and phagocytosis.

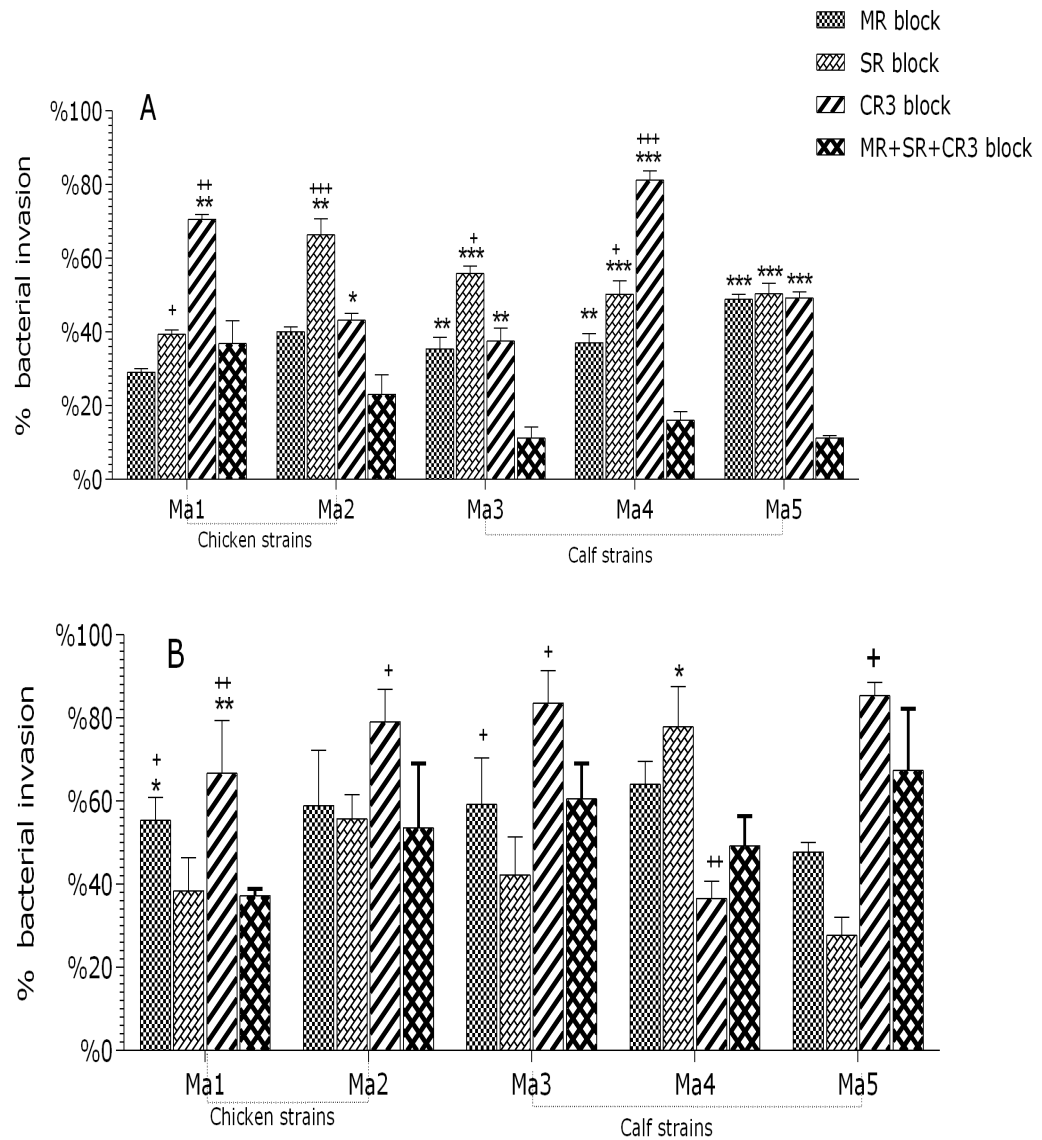


Figure 4.7: Role of mannose MR-, scavenger receptor SR- and complement CR3 receptors in invasion of *M. avium* into macrophages. (A) Human THP-1 (B) Avian HD11. Infections were carried out with (MOI 1:10) *M. avium* and CFUs of the bacteria were determined 2h post-infection. Heparin sodium salt from porcine intestinal mucosa 10 U/ml was used for blocking complement receptors CR3. Dextran sulfate sodium salt 100ug/ml for blocking scavenger receptors (SRs). α -Methyl D-Mannoside 2% was used for mannose receptors (MRs). The data are shown as the percentage relative to the control (infected cells but no block). The values shown are the means $\pm$ SE for three separate experiments with internal triplicate repeats. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08). Significant differences * p <0.05, ** p <0.01 and *** p <0.001 between simultaneous blocking of (MR+ SR +CR3) and the individual receptor blocking. Significant differences + p <0.05, ++ p <0.01 and +++ p <0.001 between MR and other receptors in THP-1 and between SR and other receptors in HD11.

4.2.4 The role of cell cytoskeleton in the bacterial invasion into the host cells.

Invasion through the different receptor mediated mechanisms can be through the different cytoskeletal component of the macrophage. To determine the role of actin and tubulin dependent mechanisms in mycobacterial invasion of THP-1 and HD11 cells, the actin depolarizing agent cytochalasin D (CD) and tubulin disrupting agent nocodazole (Noco) were used.

Prior to using the cytoskeletal inhibitors their impact on cell viability and optimal disruption of cytoskeletal components was assessed using the LDH release assay (figure 4.8 A and B) and immunofluorescence microscopy (figures 4.9 and 4.10).

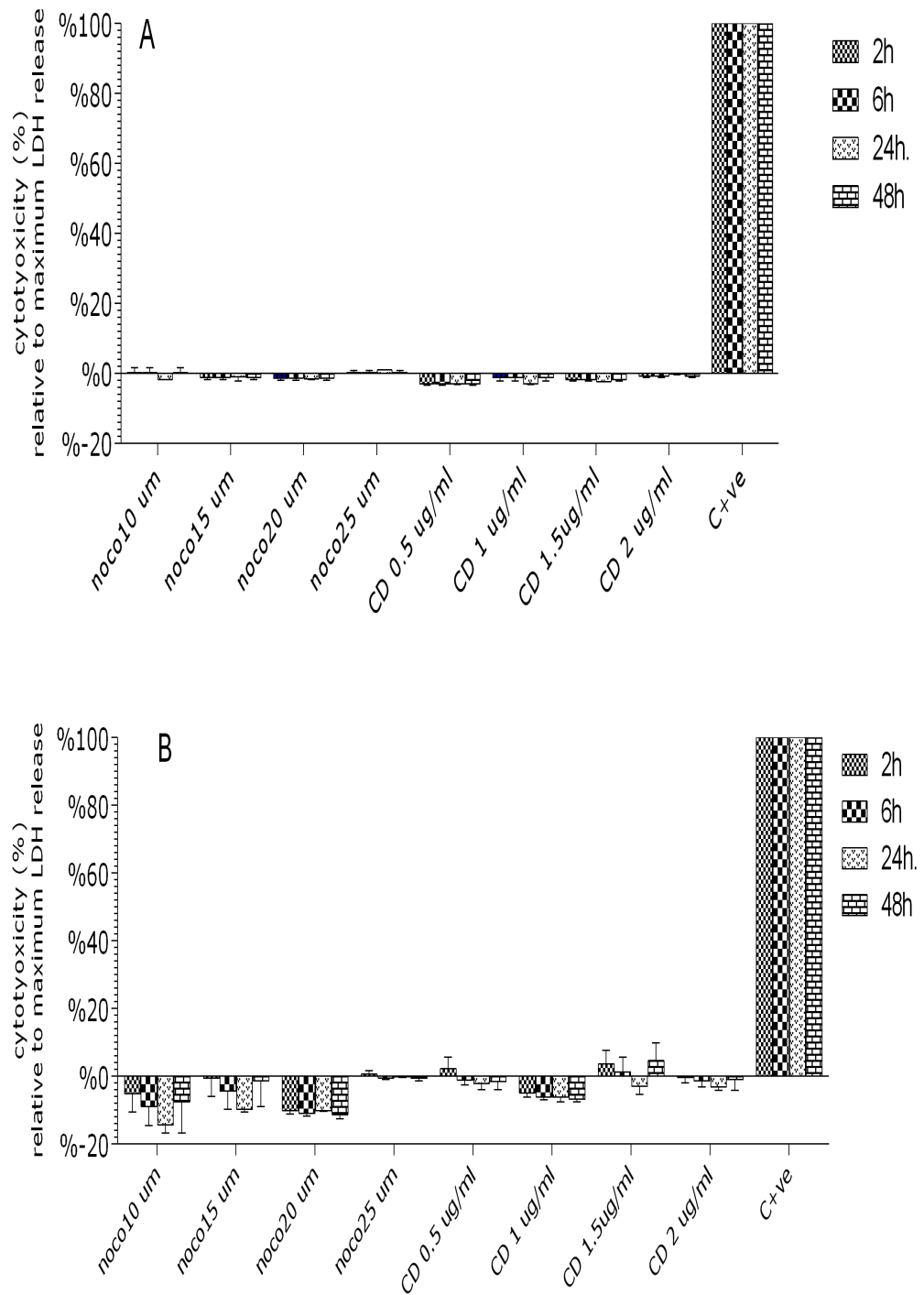


Figure 4.8: Assessment by LDH release of cytotoxicity of cytochalasin D and nocodazole on macrophages. (A) THP-1 (B) HD11 cells. The cytotoxicity (%) was measured over 48h of treatment. Values shown as mean $\pm$ SE of three independent experiments with internal triplicate repeats. The doses were chosen upon the minimum cytotoxicity and maximum disruption activity indicated by microscopy.

In cytochalasin D-treated cells, the phalloidin-stained actin was found aggregated in clumps compared to that found as an intact layer underlying the plasma membrane in the mock-infected cells. In nocodazole-treated cells, the cell tubulin revealed clumps of reorganized and redistribution of the short tubulin filament towards the centre. In mock-infected cells the intact web of tubulin was distributed throughout the cells without any signs of destruction (Figure 4.9, 4.10). Cytochalasin D at a concentration of 1µg /ml and nocodazole 20µM were selected as the best concentrations to study the effect of actin and tubulin in the invasion and intracellular survival of *M. avium* in THP-1 and HD11 cells.

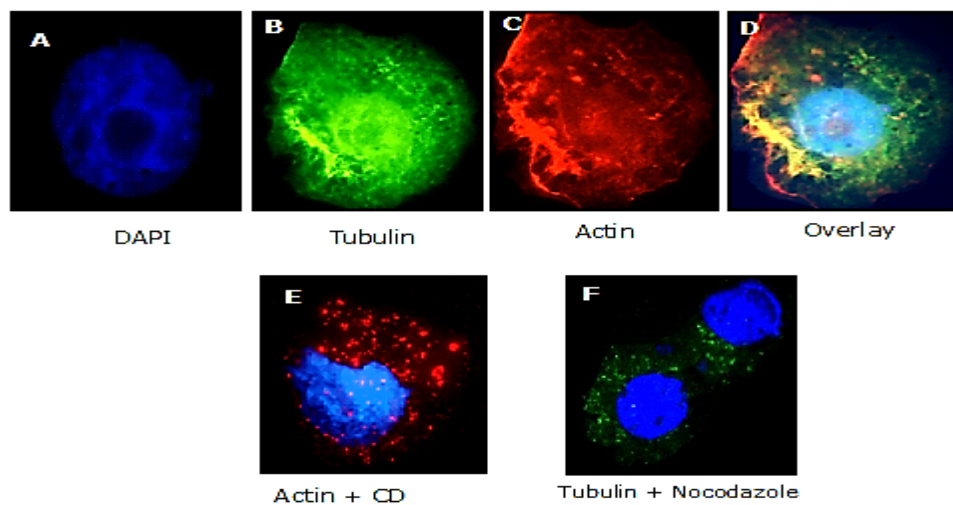


Figure 4.9: Assessment of disruption of cytoskeleton in THP-1 cells by cytochalasin D and nocodazole. Nuclear DNA visualized by DAPI (4', 6-diamidino-2-phenylindole). Tubulin visualised with goat anti rabbit IgG anti-chicken tubulin polyclonal antibody (FITC) and actin visualised with Alexa Fluor™ 546 Phalloidin. Images (A) Nuclear DNA visualized with DAPI (B) Tubulin in non-treated cell (C) Actin of non-treated cells. (D) Overlay. (E) Overlay of disrupted actin in CD-treated cells (F) Overlay, disrupted tubulin in nocodazole-treated tubulin (green). Cells were visualized using a LEICA DM 5000B Fluorescent microscopes, 63X1.25 lens

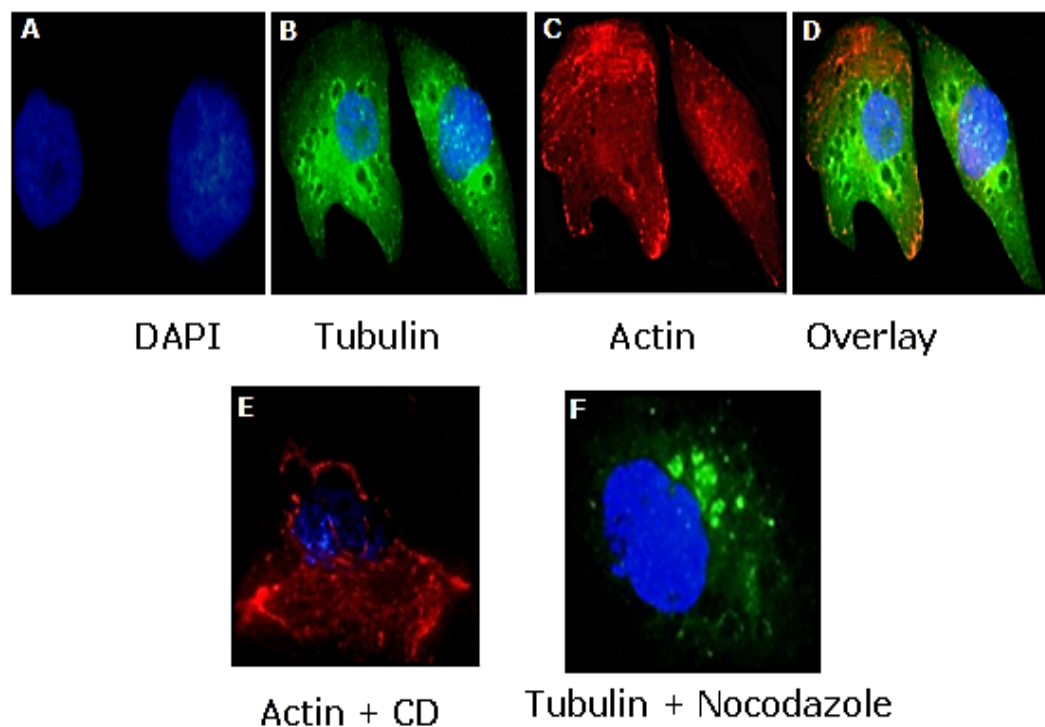


Figure 4.10: Cytoskeleton disruption in HD11. Fluorescence microscopic images **(A)** Nuclear DNA visualized by DAPI (4', 6-diamidino-2-phenylindole). **(B)** Tubulin in non-treated cell **(C)** Actin of non-treated cells. **(D)** Overlay. **(E)** Overlay of disrupted actin in CD-treated cells **(F)** Overlay, disrupted tubulin in nocodazole-treated tubulin (green). Cells were visualized using LEICA DM 5000B Fluorescent microscopes, 63X1.25 lens.

The role of actin and tubulin in invasion of *M. avium* strains of THP-1 and HD11 were then investigated. Independent blocking of actin and tubulin led to a reduction of the bacterial invasion indicating a role for the cell cytoskeleton in both human and avian cells. There were differences between the role of actin and tubulin in the bacterial invasion between the cells. The data are presented as the percentages of the bacterial invasion relative to non-inhibitor containing controls (Figure 4.11).

In THP-1 cells, the invasion of the cytochalasin D-treated cells by the bacterial strains (except Ma4) was similarly about 70%; Ma4

was less than 20%. The tubulin disrupted cells decreased the invasion rates of Ma1, Ma2 and Ma5 about 15%, Ma3 and Ma4 were reduced *ca.* 40% and about 60%, respectively. The simultaneous disruption of actin and tubulin inhibited the invasion rates over than 30% for Ma1, Ma2 and 50% for Ma3 with, about 85% for Ma4 and only 15% for Ma5.

Whereas, in cytochalasin D treated HD11, the invasion rates of Ma1, Ma2 and Ma4 were reduced about 70%, Ma3 and Ma5 were decreased about 25% and about 47%, respectively. Tubulin disruption decreased invasion rates of Ma1, Ma3 and Ma4 by about 40% and about 15% for Ma5; Ma2 was the most affected strains by tubulin disruption where the bacterial invasion rates reduced about 55%. The interaction between actin and tubulin was also investigated and the data found that the simultaneous disruption of actin and tubulin further inhibited the bacterial invasion of the cells and the invasion rates of the bacterial strains were decreased to about 35% compared to the control of untreated-infected HD11.

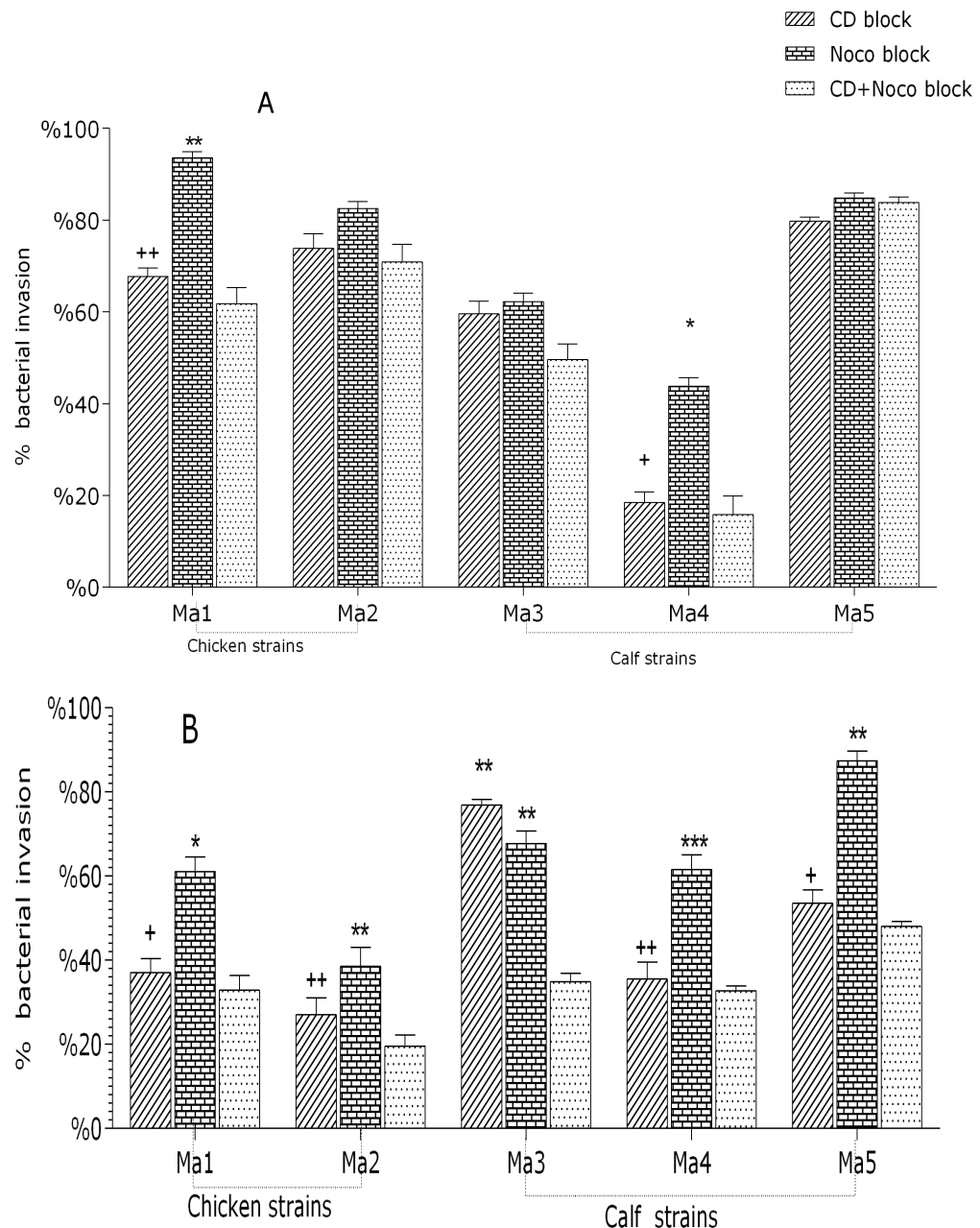


Figure 4.11: The role of cytoskeleton in invasion of *M. avium* strains Ma1-Ma5 in (A) THP-1 cells (B) HD11. (CD) cells were treated with cytochalasin D 1ug/ml. (Noco) cells treated with nocodazole 20 uM. (CD + Noco) cells were simultaneously treated with CD and Noco. The data are presented as percentage relative to the control of infected cell but in the absence of cytoskeleton disrupting agents. Infections were carried out at MOI of 10:1 in the presence of 10% v/v of host specific sera. The percentages are shown as means $\pm$ SE from three independent experiments with internal triplicate repeats. Symbol *, ** and *** refer to significant differences at $p < 0.05$ and $p < 0.01$ and $p < 0.001$, respectively between CD + Noco and other groups. Symbol + refers to significant differences at $p < 0.05$ between CD- and nocodazole-treated groups.

4.2.5. The interactions between receptors and cytoskeleton

4.2.5.1 Effects of actin and tubulin inhibitors on subsequent invasion of *M. avium* via complement receptor CR3.

To determine the link between the cell receptors and cell cytoskeletal requirements for invasion, individual cell receptors were blocked simultaneously with disruption of either actin or tubulin and compared with the non-treated control groups. In THP-1, in the presence of human serum the phagocytosis of all of the bacterial strains except Ma5 was further reduced by blocking of CR3 in the presence of actin disrupter 1 μ g/ml CD (Figure 4.12 A). In HD11 the effects of the actin disruptor were more consistent on *M. avium* invasion via CR3 in the presence of FCS; the invasion rates of Ma1 and Ma4 were significantly reduced at $p < 0.05$ and Ma2, Ma3 and Ma5 at $p < 0.01$ to lower than that seen with blocking CR3 alone (Figure 4.12 B).

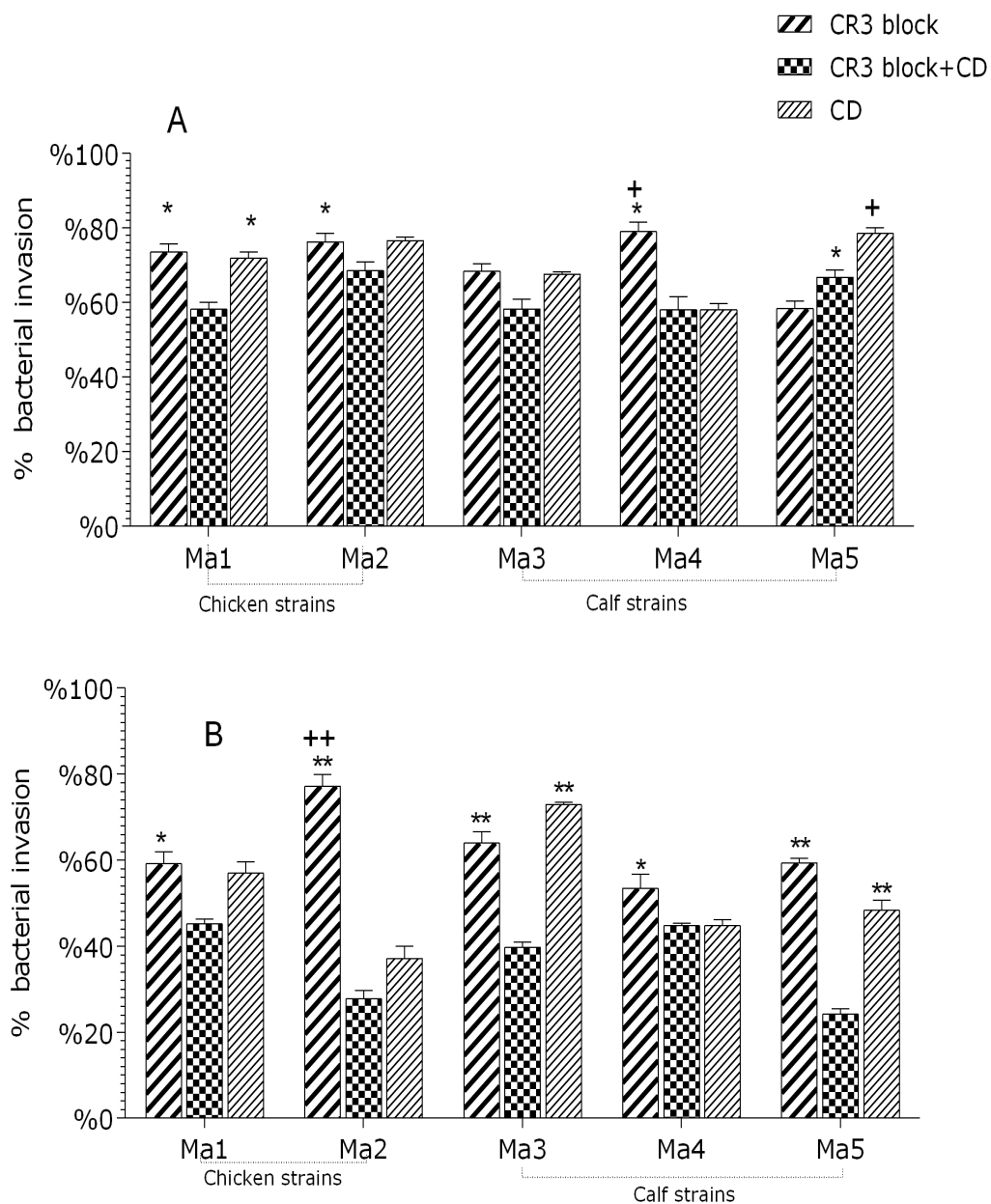


Figure 4.12: The effect of actin disruption on the subsequent invasion *M. avium* via CR3. (A) THP-1 and (B) HD11 cells. Heparin sodium salt from porcine intestinal mucosa at 10 U/ml was used for blocking complement receptors 3 CR3. Cytochalasin D 1 μ g/ml was used for actin disruption. Infections were carried out at MOI of 10:1 in the presence of 10% v/v of host specific sera. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08). The data are presented as percentages relative to the control. The percentages are shown as means $\pm$ SE from three independent experiments with three internal replicates. Significant differences $*p < 0.05$ and $**p < 0.01$ between (CR3 block + CD) and other groups and Significant differences $+ p < 0.05$ between CR3 group and CD treated groups.

To determine if the CR3 receptor is tubulin dependent in bacterial invasion of THP-1 and HD11 cells, the infections were carried out in the presence of 10% v/v of the host sera and 20 μ M nocodazole either alone or simultaneously with CR3 blocker and compared with control groups and CR3 blocked cells. The results are shown in Figure 4.13. As with the actin inhibitor, the tubulin inhibitor was able to further decrease bacterial invasion via CR3 in both cell lines with the exception of Ma2 and Ma5 in THP-1 cells which was not seen to be affected by nocodazole. These data suggest that both actin and tubulin are essential for the optimal function of CR3 and bacterial invasion for both cell lines.

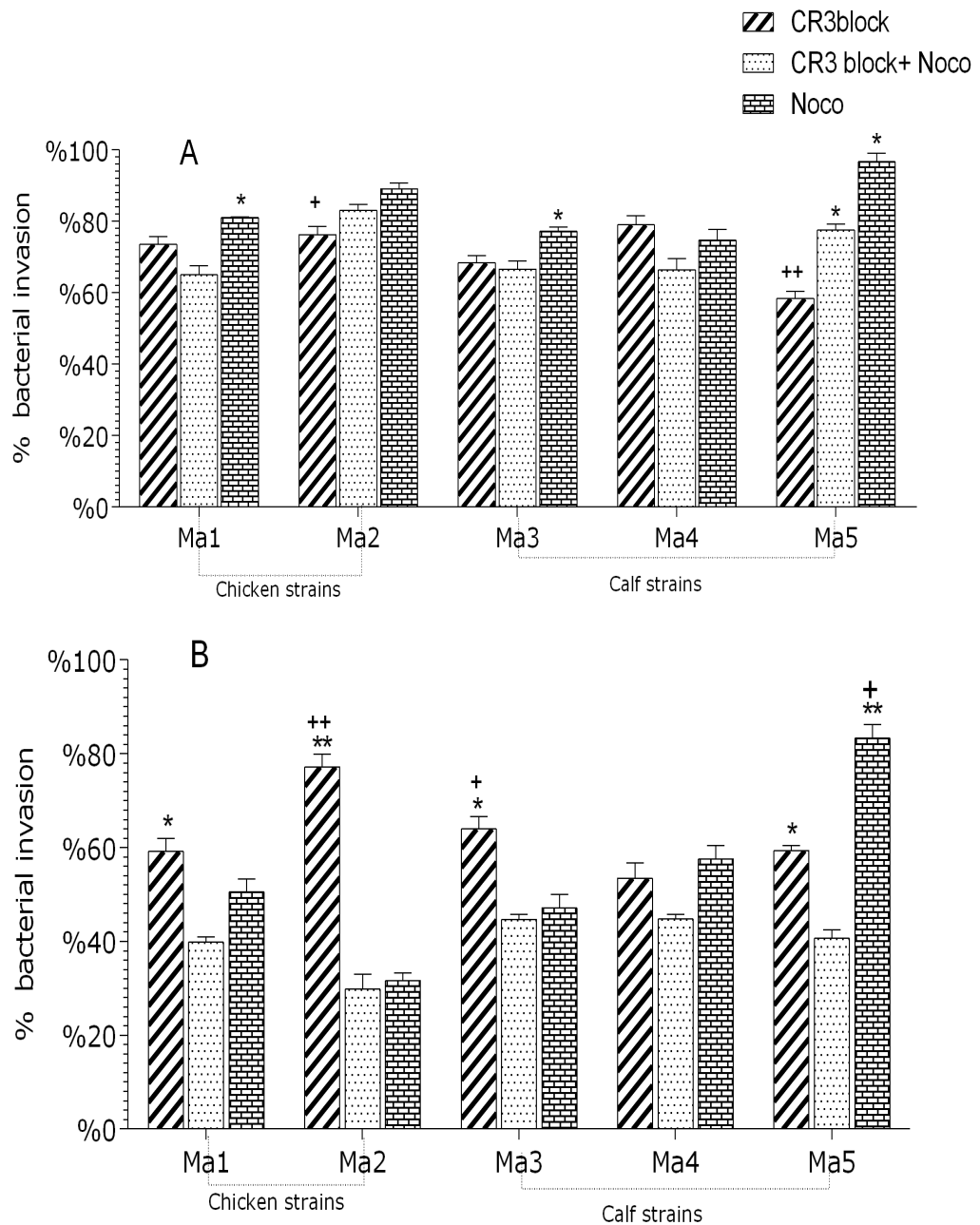


Figure 4.13: The effect of tubulin disruption on the subsequent invasion *M. avium* via CR3. (A) THP-1 and (B) HD11 cells. Heparin sodium salt from porcine intestinal mucosa at 10 U/ml was used for blocking complement receptors CR3 and nocodazole 20 μ M was used for disruption of tubulin. Infections were carried out at MOI of 10:1 in the presence of 10% v/v of host specific sera. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627-08) and Ma5 (61-3728-08). The data are presented as percentages relative to the control. The percentages are shown as means $\pm$ SE from three independent experiments with three internal repeats. Significant differences * p <0.05 and ** p <0.01 between Noco+CR3 and other groups. Significant differences + p <0.05 between CR3 group and nocodazole treated groups.

4.2.5.2 Effects of actin and tubulin inhibitors on subsequent invasion of *M. avium* via mannose receptors.

The same techniques described above (4.2.5.1) were used to determine the effects of actin and tubulin disruption on the mannose receptor mediated bacterial invasion, except that it was done in the absence of host sera. The data are shown in Figure 4.14 which indicated that actin disruption in both cell lines had a significant effect on the bacterial invasion via the mannose receptor compared to the control groups. This was particularly noticeable in *M. avium*-infected HD11 cells.

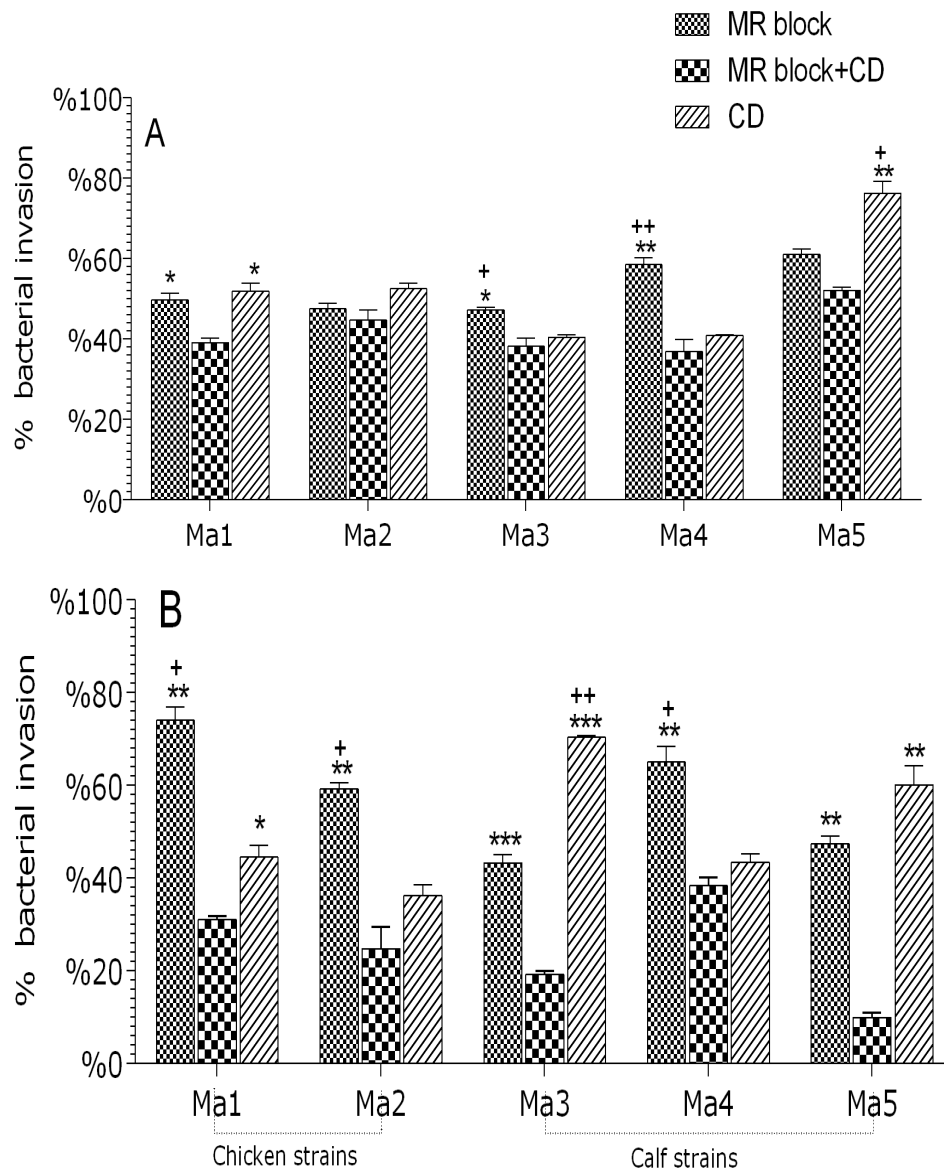


Figure 4.14: The effect of actin disruption on the subsequent invasion of *M. avium* via mannose receptor. (A) THP-1 and (B) HD11 cells. α -Methyl *D*-Mannoside 2% was used for blocking mannose receptors (MRs) and cytochalasin D 1 μ g/ml used for disruption of the cell actin. Infections were carried out at MOI of 10:1 in the absence of the host sera. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08). The data are presented as percentages relative to the control. The percentages are shown as means $\pm$ SE from three independent experiments with three internal replicates. Significant differences * p <0.05 and ** p <0.01 between (CD+MR) and other groups. Significant differences + p <0.05 between MR group and CD treated groups.

The links between cell tubulin and mannose receptors were also determined. In THP-1, the phagocytosis of the Ma2 and Ma5 strains was not further reduced by blocking of mannose receptor in the presence of tubulin disrupter compared to cells were treated with mannose receptor blocker alone. Additionally, Ma3 and Ma4 invasion was lower in the presence of tubulin blocker alone than that obtained by blocking mannose receptor and tubulin simultaneously (Figure 4.15 A). In contrast, in HD11, the link between mannose receptor and tubulin was essential for the receptor and for Ma1 and Ma2 where the bacterial invasion were significantly ($P < 0.05$) reduced by a concurrent blocking of mannose receptor and tubulin compared to mannose receptor alone. The invasion rates of strains Ma3, Ma4 and Ma5 in nocodazole-treated HD11 cells alone or with mannose receptor blocked-cells were nearly the same as that in mannose receptor plus nocodazole-treated cells (Figure 4.15 B).

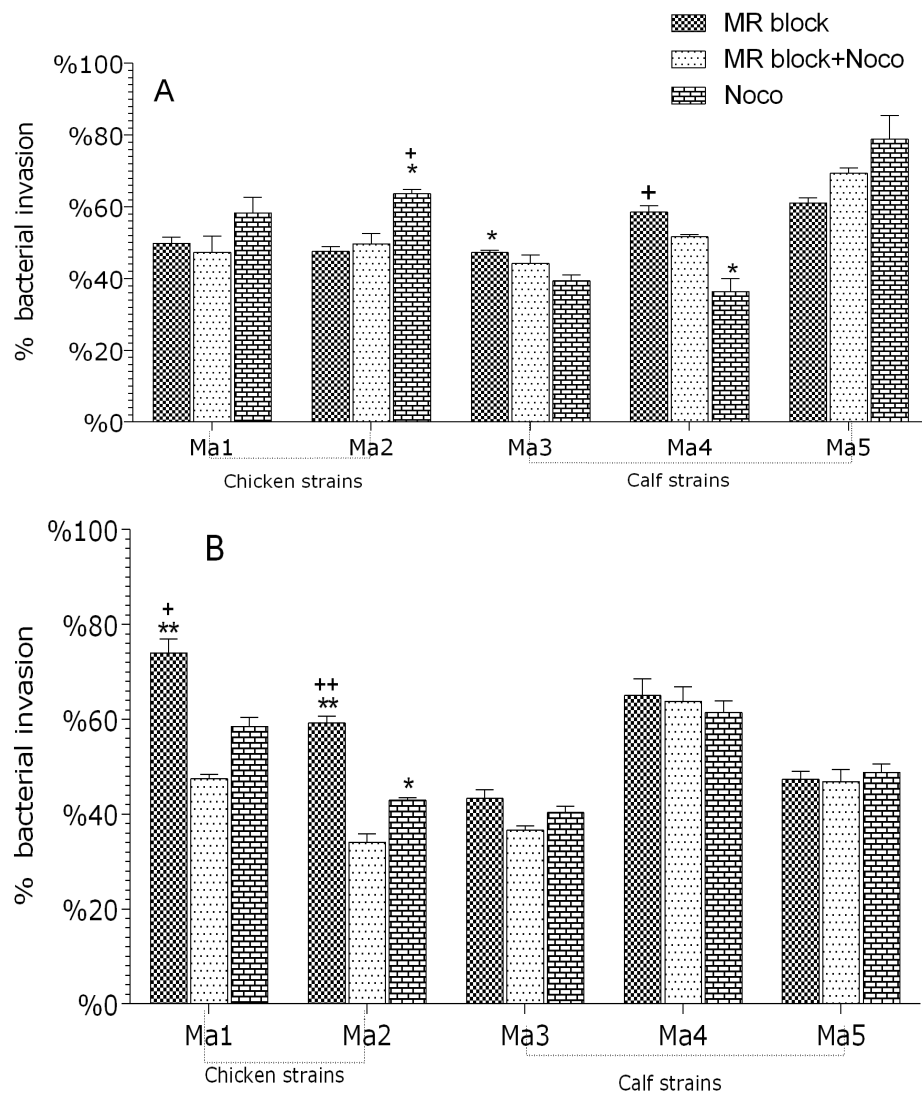


Figure 4.15: The effect of tubulin disruption on the subsequent invasion of *M. avium* via mannose receptor. (A) THP-1 and (B) HD11 cells. α -Methyl *D*-Mannoside 2% was used for blocking mannose receptors (MRs) and nocodazole 20 μ M was used for disruption of cell tubulin. Infections were carried out at MOI of 10:1 in the absence of the host sera. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08). The data are presented as percentages relative to the control. The percentages are shown as means $\pm$ SE from three independent experiments with three internal replicates. Significant differences * p <0.05 and ** p <0.01 between (Noco + MR) and other groups. Significant differences + p <0.05 between MR group and nocodazole treated groups.

4.2.5.3 Effects of actin and tubulin disruption on subsequent invasion of *M. avium* via scavenger receptors

The inhibitory effects of the concurrent blockage of scavenger receptors and actin disruption on the bacterial invasion in both cell lines were shown in Figure 4.16. The invasion of the bacterial strains of both cell lines were significantly reduced ($P < 0.05$) by simultaneous blocking of scavenger receptors and disruption of the cell actin compared to the bacterial invasion rates of THP-1 and HD11 cells which were either treated with scavenger receptors blocker or cytochalasin D alone.

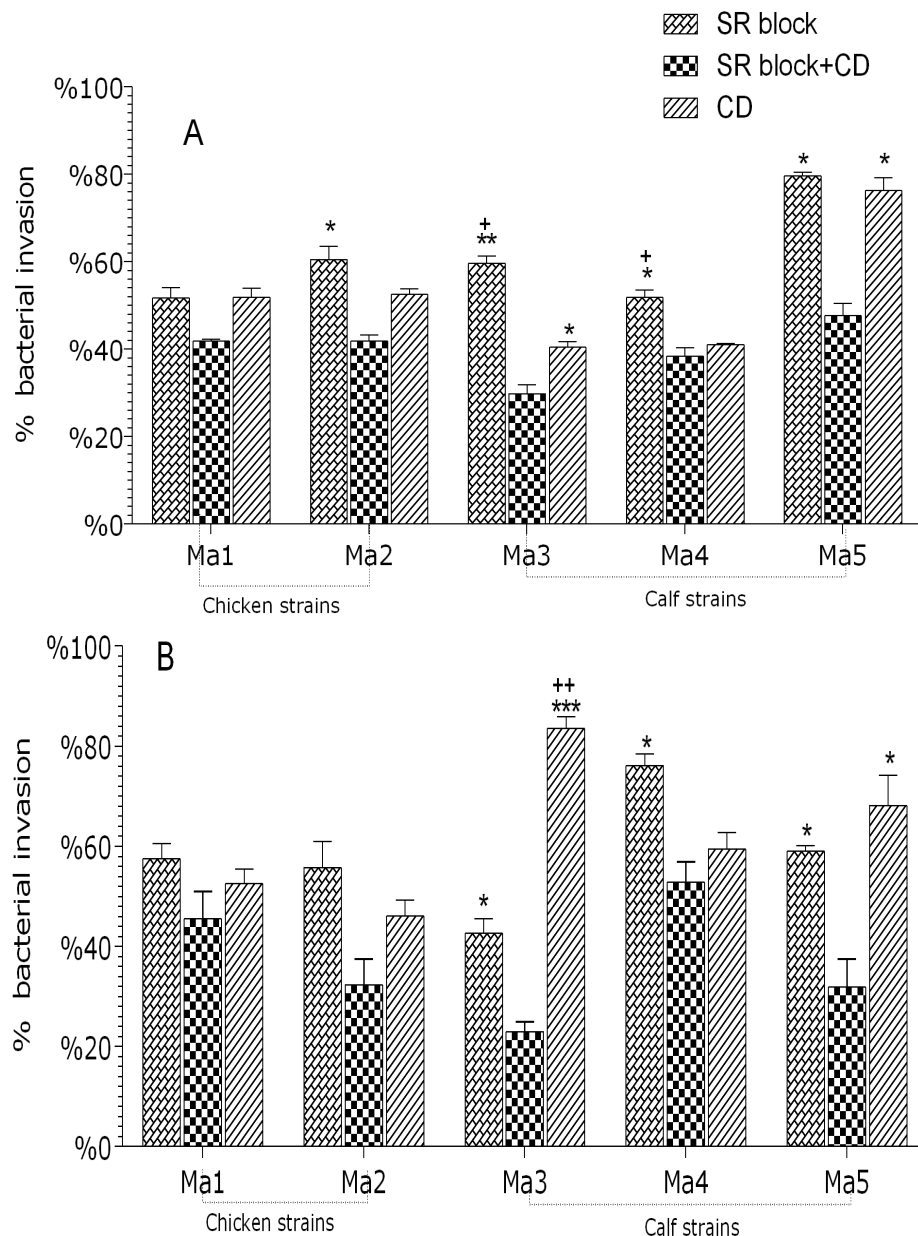


Figure 4.16: The effect of actin disruption on the subsequent invasion of *M. avium* via scavenger receptors. (A) THP-1 and (B) HD11 cells. Dextran sulfate sodium salt 100 ug/ml was used for blocking scavenger receptors (SRs) and cytochalasin D 1 ug/ml was used for disruption of the cell actin. Infections were carried out at MOI of 10:1 in the absence of sera. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08). The data are presented as percentages relative to the control. The percentages are shown as means $\pm$ SE from three independent experiments with three internal replicates. Significant differences * p <0.05 and ** p <0.01 between (actin + SR) and other groups. Significant differences + p <0.05 between SR group and cytochalasin D treated groups.

Blocking of scavenger receptors simultaneously with disruption of tubulin found cell to cell differences in the invasion rates of all of the bacterial strains. In THP-1, the mycobacterial strains were unable to invade the treated cells to the same degree when the cells were treated with scavenger receptor blocker alone (Figure 4.17 A). In contrast to THP-1, in HD11 cells no significant differences in the invasion of the bacterial strains were found between nocodazole- and scavenger receptors-treated cells simultaneously and scavenger receptor-blocked cells, with the exception of strain Ma4 (Figure 4.17 B).

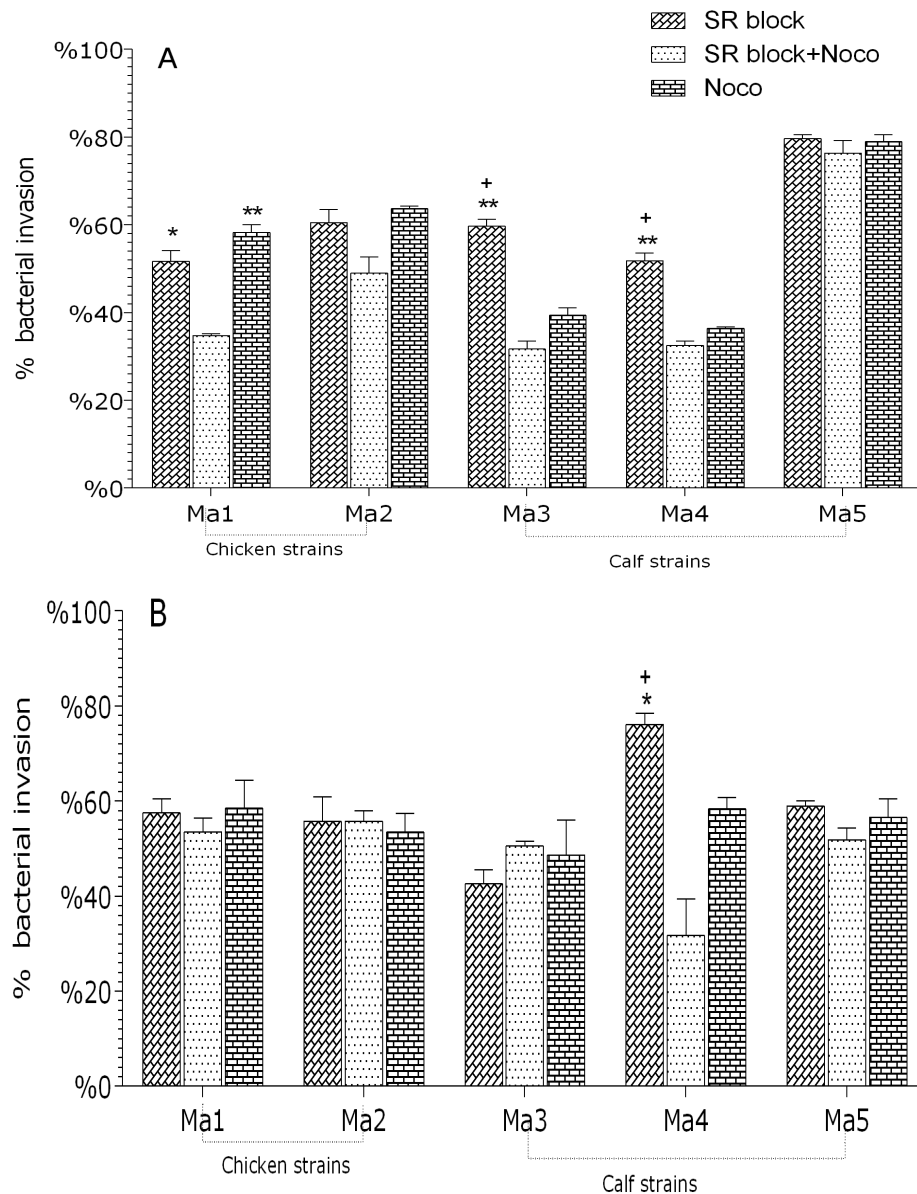


Figure 4.17: Effect of tubulin on the subsequent invasion of *M. avium*, via scavenger receptor. (A) THP-1 and (B) HD11 cells. Dextran sulfate sodium salt 100 ug/ml for blocking scavenger receptors (SRs) and nocodazole 20 μ M was used for disruption of the cell tubulin. Infections were carried out at MOI of 10:1 in the absence of sera. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559.07), Ma3 (61-3623.05), Ma4 (61-4627.08) and Ma5 (61-3728.08). The data are presented as percentages relative to the control. The percentages are shown as means $\pm$ SE from three independent experiments with three internal replicates. Significant differences * p <0.05 and ** p <0.01 between (Noco + SR) and other groups. Significant differences + p <0.05 between SR group and nocodazole treated groups.

4.3 Discussion

Invasion of host cells is a fundamental step in the pathogenesis of any intracellular survival agent and to control the pathogen infection it is important to understand in detail the mechanisms of interaction between the host and pathogen. Despite of the advances made in the understanding of the interactions between mycobacterial infection and mammalian host cells, the exact mechanisms of the bacterial invasion and the links between the cell surface receptors and the cell cytoskeleton are not fully understood. Mycobacteria invade host cells, particularly macrophages, in different ways (Ernst, 1998). *In vitro* studies found that *M. avium* invasion of host macrophages was enhanced in the presence of the host sera (Bermudez et al., 1991). Coated bacteria with the complement component C3b were rapidly ingested via CR3 (Hirsch et al., 1994). Additionally, bacteria were found to enter host cells in the absence of the sera depending on the mannose and scavenger receptors (Ernst, 1998). However, it is widely accepted that actin is essential for bacterial invasion. Recently, tubulin-dependent invasion theories are gaining importance (Silva et al., 2013).

In this study, the initial invasion of *M. avium* in human and chicken macrophages was investigated in the presence of host sera. The data showed that invasion of *M. avium* into THP-1 and HD11 cells depends significantly on the presence of serum. Schorey et al. (1997) reported that the invasion of human macrophages by *M.*

tuberculosis and *M. avium* was significantly enhanced by human serum. Human serum is very important for *M. avium* to invade THP-1 compared to the other sera which restricted the bacterial invasion. In contrast, *M. avium* invasion into HD11 was significantly enhanced in the presence of animal sera compared to the human sera and serum free RPMI.

The variation between the effects of the different sera may be due to the differences in the concentration of the complement proteins in each serum or even is probably due to the host species-specific differences in the structure of the complement protein C3. The concentrations of C3 in FCS are very low to undetectable (Barta and Hubbert, 1978). Human serum contains a much higher concentration at between about 0.75-1.35mg/ml (Linscott and Triglia, 1980) and chicken serum contains C3 at approximately 0.5 mg/ml (Mavroidis et al., 1995). The chicken proteins Clq (Yonemasu and Sasaki, 1986), factor B (Kjalke et al., 1993), and C3 (Laursen and Koch, 1989) are thought to be similar in function and structure to that found in the mammalian sera, whereas it has been claimed that the chicken C3 exists in three different molecular forms (Laursen and Koch, 1989). There is thus scope for differences in component concentrations and steric hindrance involved in the results found. Furthermore, it has been found that the physiological levels of IFN- γ in human sera is (11.4 pg/ml) (Davoodi et al., 2012); IFN- γ inhibits function of complement receptor in human monocyte-derived macrophages and significantly reduces

phagocytosis of *Mycobacterium leprae* (Schlesinger and Horwitz, 1991). It has also been reported that IFN- γ down-regulates the expression of mannose receptor in murine macrophages (Harris et al., 1992, Stahl and Ezekowitz, 1998, Stein et al., 1992). However, there is no evidence to prove that the human IFN- γ has any effect on expression of phagocytic receptors in avian macrophages but upon the published studies it is assumed to have the same down regulatory effect on mannose and complement receptors in HD11 cells.

This study identifies significant variation between results obtained with fresh and frozen sera for bacterial invasion. Invasion of THP-1 and HD11 cells were significantly enhanced in the presence of fresh human and chicken sera, whereas no significant differences were observed in the bacterial invasion for HD11 in the presence of fresh or cryopreserved FCS. This is likely due to the denaturation of C4 as a result of freezing effects on the sera (von Zabern and Gigli, 1982). FCS contains very low concentrations of C4 compared to human and chicken sera (Barta and Hubbert, 1978). It has also been found that the cryopreservation of sera affects MASP-1 and MASP-2 (enzymes activated by mannose-binding lectin and required for activation and cleavage of C4 and C2 to form C4bC2b a C3 convertase) which in turns adversely affects C4 cleavage (Presanis et al., 2004).

The complement system can be activated through two pathways, the classical activation of the complement proteins through binding

of C1q binding to antibodies (Mold et al., 1999) and alternative pathways which does not need the antigen-antibody complex to be activated but, instead C3b binds directly to the bacterial surface (Rus et al., 2005, Bartłomiejczyk et al., 2014, Thurman and Holers, 2006, Janeway et al., 2001). The data in this study indicated that all the bacterial strains were able to activate both classical and alternative pathways in human sera, although, the differences between the EGTA- and EDTA-treated sera were not significant. These findings are in agreement with that reported by Schorey et al., (1997) when they found that *M. tuberculosis*, *M. leprae*, and *M. avium* activate complement via the alternative pathway even in the absence of C4. The inhibitory effects of EDTA and EGTA were more consistent with human sera than with FCS and CHS probably as a result of the lower concentrations of the complement proteins in the animal sera than those found in human sera.

M. tuberculosis can invade mammalian cells via mannose-, scavenger- and complement receptors CR3 (Ernst, 1998). The role of these receptors in *M. avium* has not been described; this work showed that the invasion rates of the bacterial strains were significantly reduced by blocking mannose-, scavenger- and complement receptors CR3. However, there were differences between the roles of the receptors in the bacterial invasion with mannose receptor-mediated bacterial invasion being the predominant route for invading THP-1 cells. These data agree with those reported for the invasion of pathogenic strains of *M.*

tuberculosis in human monocyte-derived macrophages which greatly depend on mannose receptors (in the absence of human sera) and complement receptor in the presence of human sera (Schlesinger, 1993, Zimmerli et al., 1996)

In HD11 cells, the data reveal that the scavenger receptor-mediated invasion is the most important mechanism utilized by the all of the bacterial strains except Ma4. In contrast, complement receptor CR3-mediated appeared to be dominant in the invasion of Ma4 in to HD11 in the presence of sera. The use of scavenger receptors by the bacterial strains in the HD11 cells are in line with studies which indicated that the mycobacterial burden of *M. bovis* BCG in liver and spleen in Cd36^{-/-} (SR B^{-/-}) mice was reduced to 83% compared to wild type Cd36^{+/+} mice (Hawkes et al., 2010). In addition, it has been seen that *M. tuberculosis* alternatively use scavenger receptors to invade the host cells, when the bacterial invasion via complement receptors and mannose receptors was inhibited (Zimmerli et al., 1996).

Simultaneous blocking of mannose-, scavenger- and complement receptor CR3 did not result in complete inhibition of bacterial invasion in either cell line suggesting that the cell surface receptors are not working independently or other receptors may exist. Macrophages surface receptors depend on each other for optimal function when the target is a microorganism like mycobacteria with

different ligands that can interact with different receptors on the host cells at the same time (Ernst, 1998).

The receptors and cytoskeletal mechanism involved in invasion may have an impact on the compartment in which the bacteria reside post-invasion and that compartment's maturation state. To determine the role of the cell cytoskeleton in *M. avium* invasion of THP-1 and HD11 cells, actin and tubulin depolarizing agents were used. The data showed that both actin and tubulin are important for internalization of *M. avium* of both cell lines. The role of actin appears to be more dominant than tubulin. The reductions in the bacterial invasion due to actin and tubulin disruption likely reflect their role in receptor mediated uptake by macrophage by the mannose-, scavenger- and complement receptors as described in mammalian systems. Remodelling of actin is required for optimal internalization of particles by mammalian macrophages (Al-Jarallah et al., 2014, Newman et al., 1991, Allen and Aderem, 1996, Defife et al., 1999). It has also been seen that the disrupted tubulin inhibits particle internalization into human macrophages via complement and scavenger receptors (Sulahian et al., 2008); likewise we observed similar results with avian cells.

The link between the specific cell receptors and cell cytoskeletal requirements for the bacterial invasion of both cell lines were investigated. The data showed that bacterial invasion was decreased by both cytoskeleton and receptor inhibitors. The data is suggestive

that a combination of receptors and cytoskeletal remodelling is used during infection such that no single combination of blocking agents totally inhibits invasion. For instance, a combinatorial effect of cytochalasin D or nocodazole on CR3-mediated invasion of Ma5 into THP-1 and nocodazole-affected CR3-mediated phagocytosis of Ma2 by THP-1 cells was not additive, but the invasion was lower than that obtained by blocking of cytoskeleton alone. Nocodazole and mannose receptor blocker were not synergistic, implying that the bacterial invasion via mannose receptor is not a tubulin-dependent mechanism. These findings are in agreement with that reported by Garcia-Perez et al. (2011) who found that the invasion of *M. tuberculosis* of human fibroblasts does not require tubulin reorganization. To our knowledge, the inhibitory effects of combinatorial treatments with actin- and tubulin-depolarizing agents and receptor competitive blockers on the mycobacterial invasion into the host cells have not been investigated before. The non-additive effects of a simultaneous blocking of individual receptor and cell cytoskeleton on bacterial invasion of THP-1, but not HD11, are probably due to the ability of the bacteria to interact with multiple different cell surface receptors to promote invasion. The differences between the cells might be due to the structural differences between human and avian cells, since it has been seen that the mammalian multigene families encoding actin cytoskeleton are more complex and larger than those in chicken cells (Chang et al., 1984)

suggesting that requirement for cytoskeleton remodelling during bacterial invasion may differ between avian and mammalian cells.

In summary *M. avium* has different routes to invade the host cells, serum factors are host-specific and the bacterial strains are able to activate the complement system in both classical and alternative pathways in the absence of antigen-antibody complexes. Data on the effects of blocking agents reveal that both mannose- and scavenger receptors have similar effects on the bacterial invasion in both THP-1 and HD11, but indicated specific effects for CR3. *M. avium* invasions seem to be more dependent on actin than tubulin for both cell lines. The cell receptors cooperate with each other during bacterial invasion. The inhibitory effects of combinatory blocking of receptors and cell cytoskeleton are synergistic on the bacterial invasion of both THP-1 and HD11 cells. Since both mannose- and scavenger receptors are dominant for *M. avium* invasion of HD11 cells it would be valuable to determine their effect on the bacterial survival in HD11 and also to detect the acidity of the phagosomes at different times points post-infection over 48h post-infection. This work is described in the chapter six.

5.0 The role of innate antimicrobial mechanisms in the survival of *M. avium* in host cells

5.1 Introduction

Macrophages are the most effective phagocytic cells involved in the innate immune response against mycobacterial infections (Van Crevel et al., 2002). Nitric oxide and reactive oxygen species have been widely studied as antimicrobial components of the murine immune system during mycobacterial infection (Chan et al., 2001). Murine macrophages kill both *M. tuberculosis* and *M. bovis* BCG by nitric oxide dependent mechanisms (Jagannath et al., 1998, Long et al., 1999, MacMicking et al., 1997, Chan et al., 1995, Ling et al., 2013). The infected macrophages can also produce reactive oxygen species (ROS) including superoxide anion, hydrogen peroxide, hydroxyl radical and singlet oxygen which are commonly regarded as bactericidal and extremely toxic to intracellular pathogens (Bartos et al., 2004, Edwards et al., 2001, Nathan and Shiloh, 2000). Activated macrophages also produce antioxidants to protect themselves from the toxic effects of their own ROS and reactive nitrogen intermediates (Venketaraman et al., 2003b). Glutathione is an antioxidant produced in huge amounts in *M. bovis* BCG-infected murine macrophages (Seres et al., 2000). Glutathione reacts with nitric oxide to form s-nitrosoglutathione which is also bactericidal (Deneke and Fanburg, 1989, Jagannath et al., 1998, Morris and Hansen, 1981). It has been shown that nitric oxide exerts its

bactericidal activity when iNOS is co-localized with the phagosome containing ingested pathogens (Miller et al., 2004).

While extensive studies have been carried out with mammalian macrophages as a model system to study the effects of innate antimicrobial mechanisms on *M. tuberculosis* and *M. bovis* BCG to our knowledge no studies has been reported on the intracellular killing mechanisms of avian macrophages against *M. avium* infection. This chapter describes the work to investigate in greater detail the roles of nitric oxide and ROS in intracellular survival of *M. avium* in chicken macrophages compared to human macrophages.

5.2 Results

5.2. 1. The role of nitric oxide in intracellular survival of *M. avium* in infected HD11 cells.

Preliminary data reported in chapter 3 indicated a relationship between induction of nitric oxide and the intracellular survival of *M. avium* within infected HD11 cells. To verify that the reduction within the bacterial survival was due to nitric oxide production, a nitric oxide stimulator (recombinant chicken IFN- γ , rchIFN- γ) and inhibitor (N^G-monomethyl-L-arginine, monoacetate, *L*-NMMA) were used in cell survival assays. The HD11 cells treated by rchIFN- γ were able to induce increased levels of nitric oxide at 24 and 48h post-infection compared to untreated cells when infected with all *M. avium* strains ($p < 0.05$) (Figure 5.1 A and B). In contrast, nitric oxide production induced by *M. avium*-infected HD11 was nearly completely inhibited when cells were treated with *L*-NMMA (Figure 5.1 C).

The viable numbers of intracellular *M. avium* were assessed at different time points post-infection for cells treated with rchIFN- γ , inhibitor *L*-NMMA and in mock treated cells. The reduction in the number of the viable *M. avium* correlated with an increased level of nitric oxide production while survival of bacteria increased in cells with reduced induction of nitric oxide (Figure 5.2). There were some minor strain to strain differences observed in the intracellular survival of the specific strains. However, the results clearly suggest that nitric oxide plays a significant role in intracellular killing of the *M. avium* within HD11 cells.

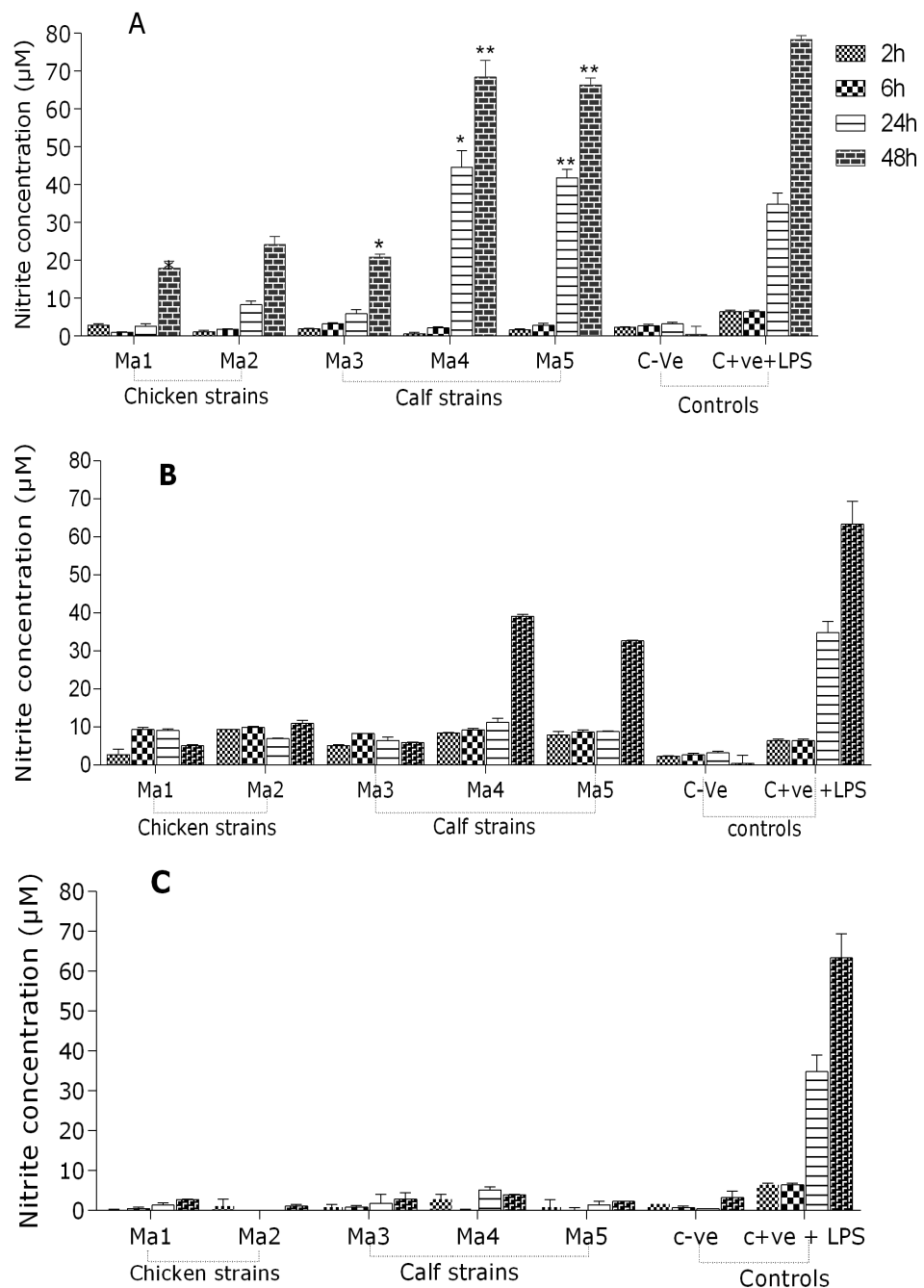


Figure 5.1: Nitric oxide production by *M. avium* infected HD11 cells. (A) Cells treated with rchIFN- γ at 100 U/ml (B) Non treated HD11 cells. (C) HD11 cells treated with L-NMMA at 1 mM. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08). Data shown are mean concentrations $\pm$ SE for three separate experiments with internal triplicate repeats. Significant differences $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$, between rchIFN- γ (A) and non-treated cells (B) at each time point post infection reported by each mycobacterial strain.

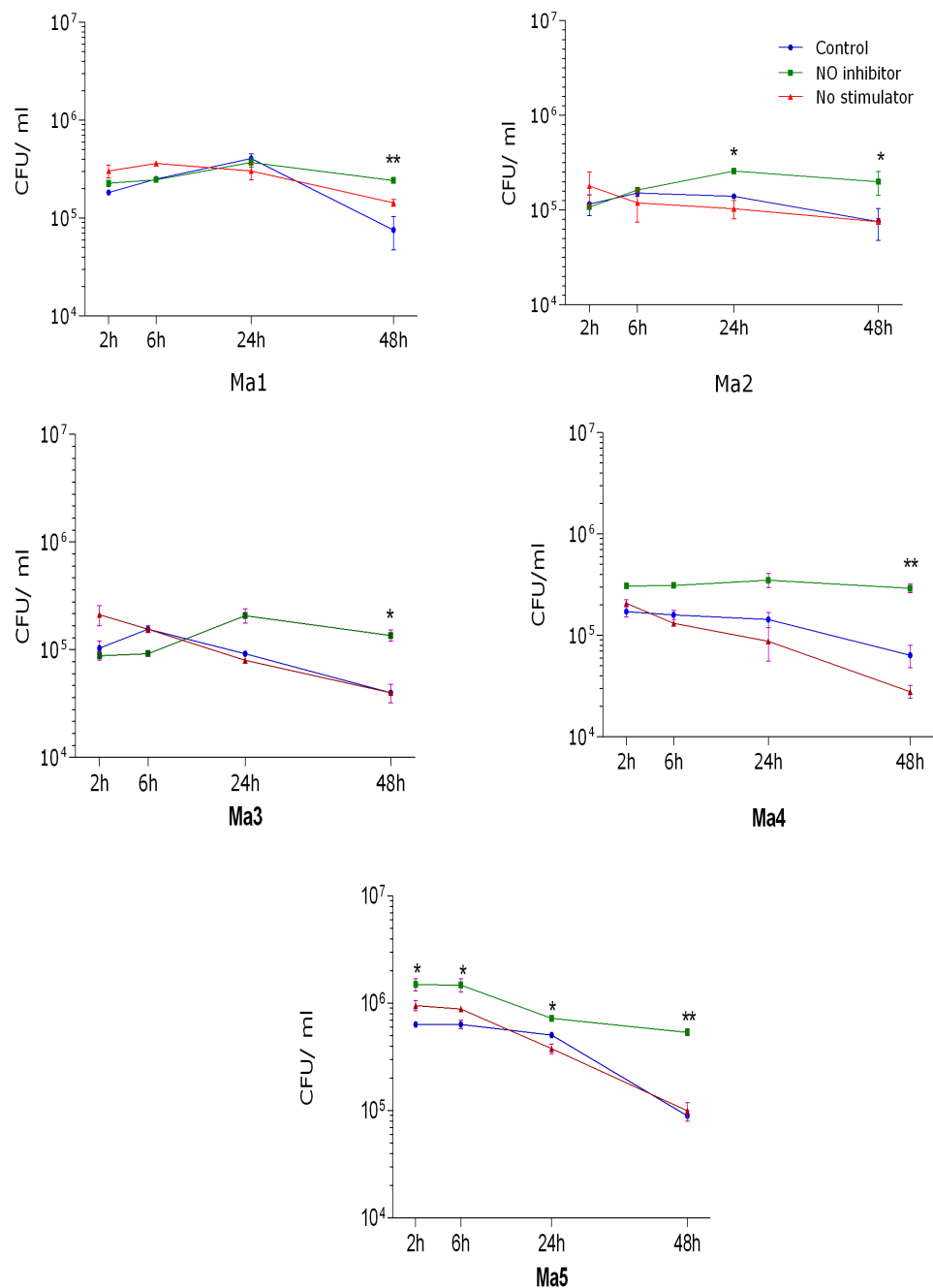


Figure 5.2: Effect of nitric oxide inducer and inhibitor on *M. avium* survival in HD11 cells. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08). Samples taken at cells at 2h, 6h, 24h and 48h post-infection. ($\blacktriangle$) cells treated with rchIFN- γ at 100 U/ml, ($\blacksquare$) HD11 cells treated with L-NMMA at 1mM ($\bullet$) and negative control untreated cells. The values shown are the means $\pm$ SE for three separate experiments with internal triplicate repeats. * $p < 0.05$, ** $p < 0.01$ - significant differences between survival in the rchIFN- γ and L-NMMA treated cells.

5.2.1.1 Co-localization of *M. avium* with iNOS in infected HD11 cells

It has been suggested that mycobacteria disrupt the co-localization and production of nitric oxide to facilitate their survival in the macrophage environment. To determine if this was the case immunofluorescent techniques were performed to detect the percentage of co-localized bacteria within iNOS at different time points post-infection. Strains Ma1 and Ma4 were selected as representatives of the strains based on their differential ability to induce nitric oxide. The iNOS staining (red colour) was dense around the co-localized bacteria (yellow) after merging compared to the diffuse red staining of iNOS around the non-co-localized bacteria (green) (Figure 5.3 and 5.4).

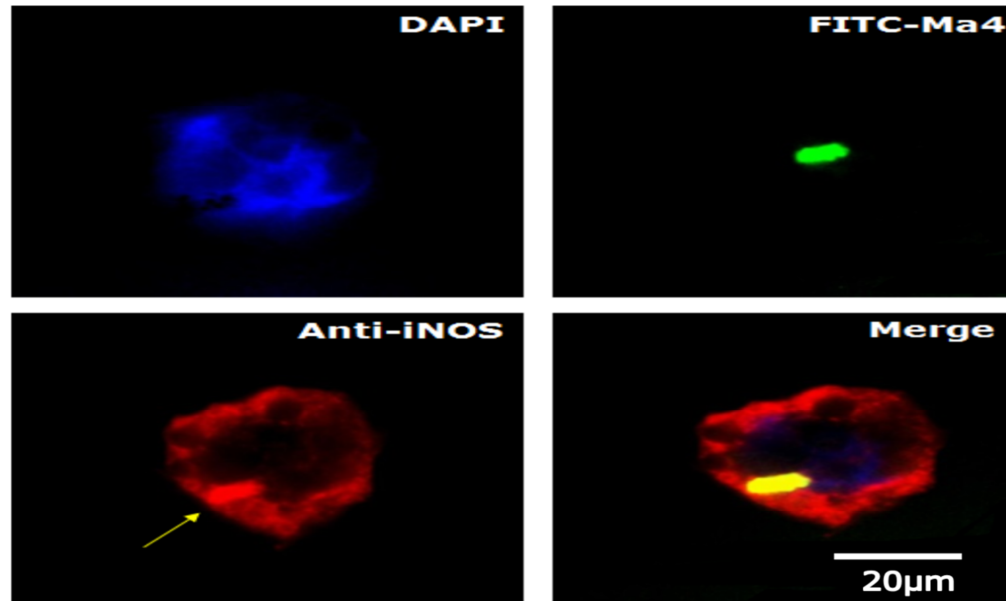


Figure 5.3: Representative fluorescence micrograph of *M. avium* co-localized with iNOS in HD11. Cells were stained with anti-iNOS antibodies followed by a secondary antibody conjugated to Alexa 568. Yellow arrow indicates condensed nitric oxide around the *M. avium*. 50 fields in cover slips were counted in triplicate wells of duplicate experiments for each strain

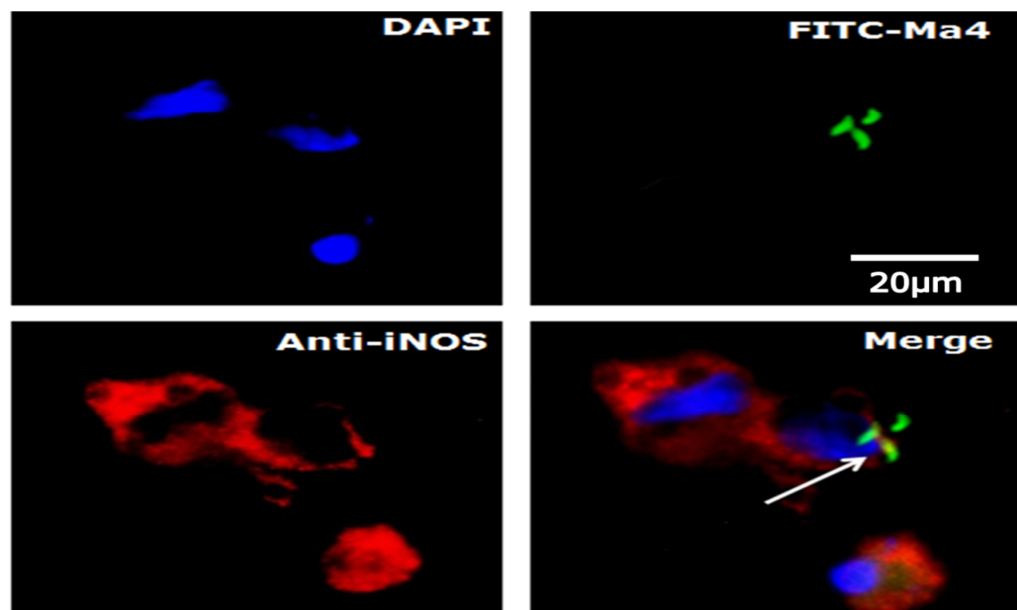


Figure 5.4: Representative fluorescence micrograph of *M. avium* non-co-localized with iNOS in HD11. Cells were stained with anti-iNOS antibodies followed by a secondary antibody conjugated to Alexa 568. White arrow indicates green coloured *M. avium* did not co-localized with iNOS. 50 fields in cover slips were counted in triplicate wells of duplicate experiments for each strain.

The numbers of the bacteria co-localized with iNOS were quantified and compared with non-co-localized bacteria. The data are presented as percentages and at different time points post-infection (Figure 5.5). There were strain to strain differences within the co-localized percentages; the rate of co-localized Ma1 at 2h and 6h was very low with a diffuse staining of iNOS found within the infected cells. The rates of iNOS-co-localized with Ma1 increased by time to reach nearly 50% by 24h post-infection and 75% by 48h after infection. In contrast, Ma4-infected HD11 cells showed increased rates of co-localization during early times points post-infection and reached over 70% by 24h post-infection (Figure 5.5). A correlation between the bacterial survival and iNOS-co-localization rates was also detected, the analysed data showed a strong negative correlation at $p < 0.001$ between the numbers of viable bacteria of Ma4 and Ma4-iNOS-co-localization rates in infected HD11 over 48h post-infection, while the correlation between the viable numbers of Ma1 and the Ma1-iNOS-co-localization rate was not significant ($p > 0.05$). The data suggest that Ma1 interferes with iNOS co-localization. The potential exists that this mis-localisation may protect the bacteria from subsequent nitrosative damage.

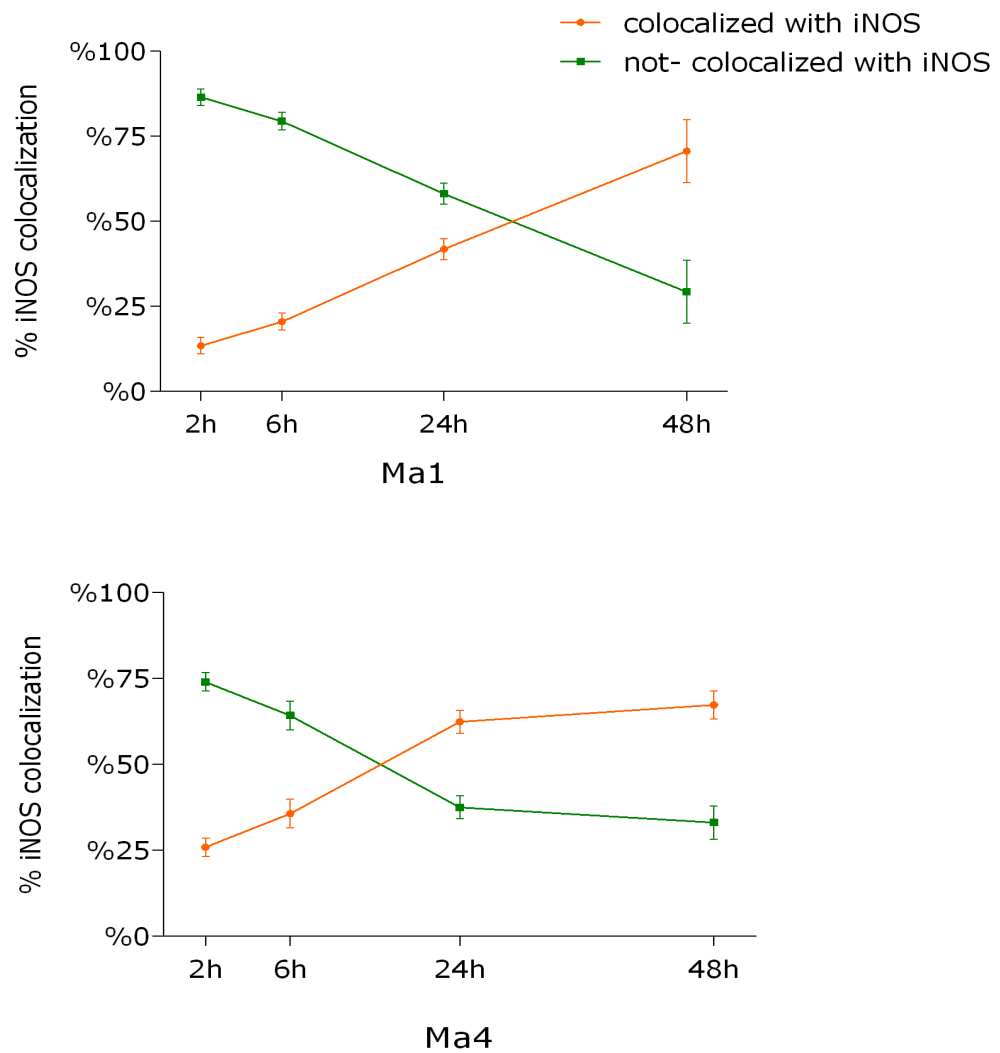


Figure 5.5: Quantitative analysis of iNOS co-localization with *M. avium* strains Ma1 and Ma4. Mycobacteria co-localized with iNOS were counted over different time points post infection (2, 6, 24 and 48h) in infected HD11 macrophages. (■) symbol refers to the co-localized *M. avium* with iNOS (■) refers to non-co-localized *M. avium* with iNOS. Data are shown as mean $\pm$ SE. The percentages of the co-localized bacteria with iNOS were scored by examining 50 randomly selected microscopic fields of cover slips in triplicate wells of duplicate experiments for each strain.

5.2.2 The role of ROS in intracellular survival of *M. avium* in infected host cells.

A correlation between level of ROS induced by infection and increases in the intracellular survival of *M. avium* within the infected

cells was observed (chapter three). To determine if the observed increases in survival were the result of difference in ROS production we repeated the infection assays with an inhibitor, inducer and mock treatment. These assays used phorbol-12-myristate-13-acetate a stimulator of ROS (PMA) and the inhibitors, *N*-acetyl-*L*-cysteine (NAC) and diphenyleneiodonium chloride (DPI). The experiments were run and compared to the non-treated control group.

The data show a clear reduction of ROS in the inhibitor group compared to both the inducer and untreated but infected cells for both THP-1 and HD11 cells at all time points (Figure 5.6 and 5.7).

Some strain to strain variation was observed in the induction of ROS by *M. avium* in both HD11 and THP-1 but the variation was less marked than for nitric oxide (Figure 5.6a and 5.7a).

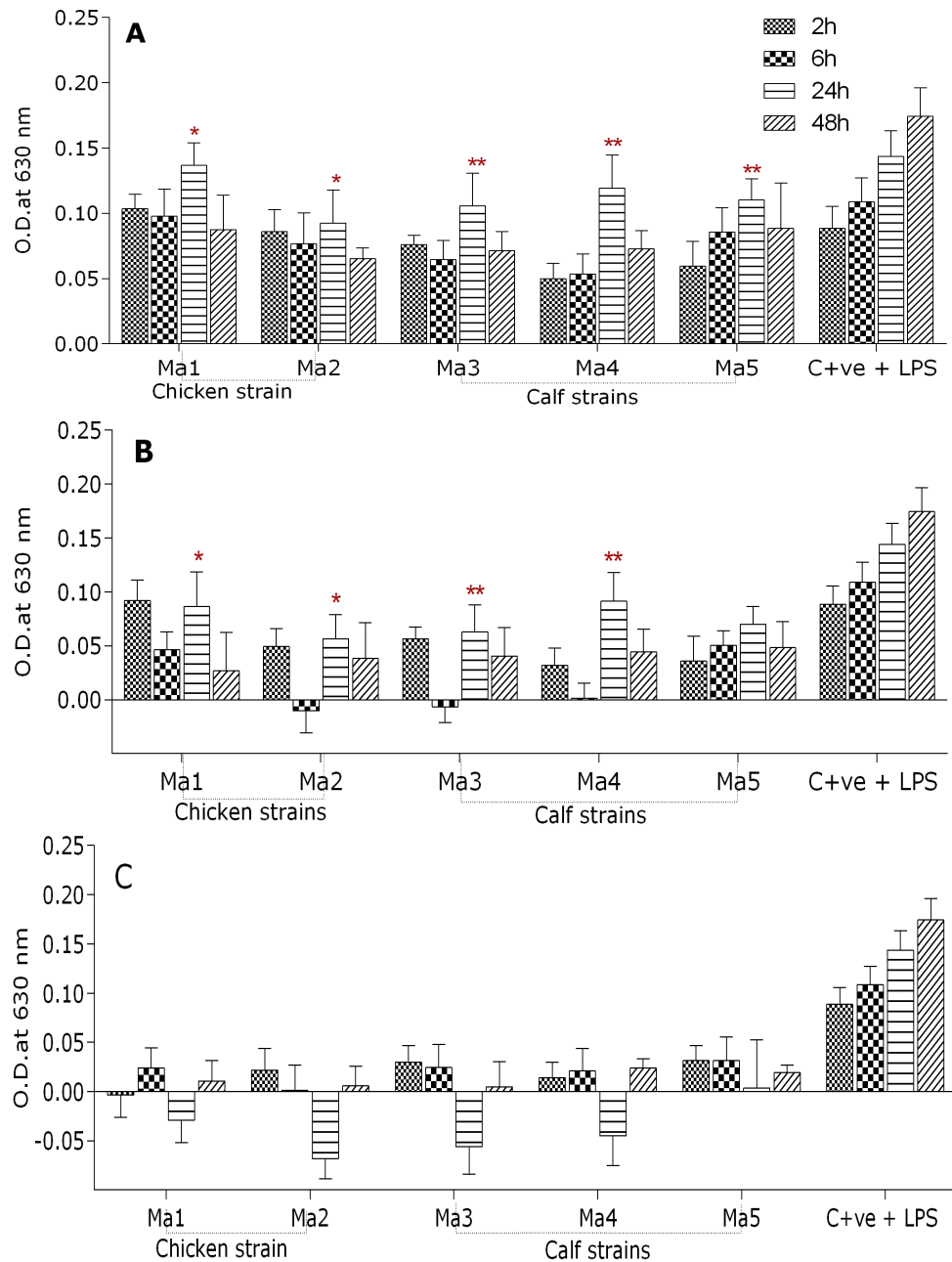


Figure 5.6: ROS production by *M. avium* infected THP-1 cells. ROS level is expressed as increase of the optical density at 630 nm (OD 630 nm) above negative control **(A)** Stimulator groups were treated with phorbol-12-myristate-13-acetate PMA 100 ng/ml **(B)** Basic group; non-treated THP-1 cells. **(C)** Inhibitor group cells were treated with *N*-acetyl-*L*-cysteine NAC 10mM and diphenyleneiodonium chloride DPI 10uM. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08). Values shown are mean $\pm$ SE of three separate experiments with internal triplicate repeats. *M. avium* strains: Significant differences $*p < 0.05$ and $**p < 0.01$ in the ROS levels changes between time points (6h and 24h post-infection) reported by each mycobacterial strain.

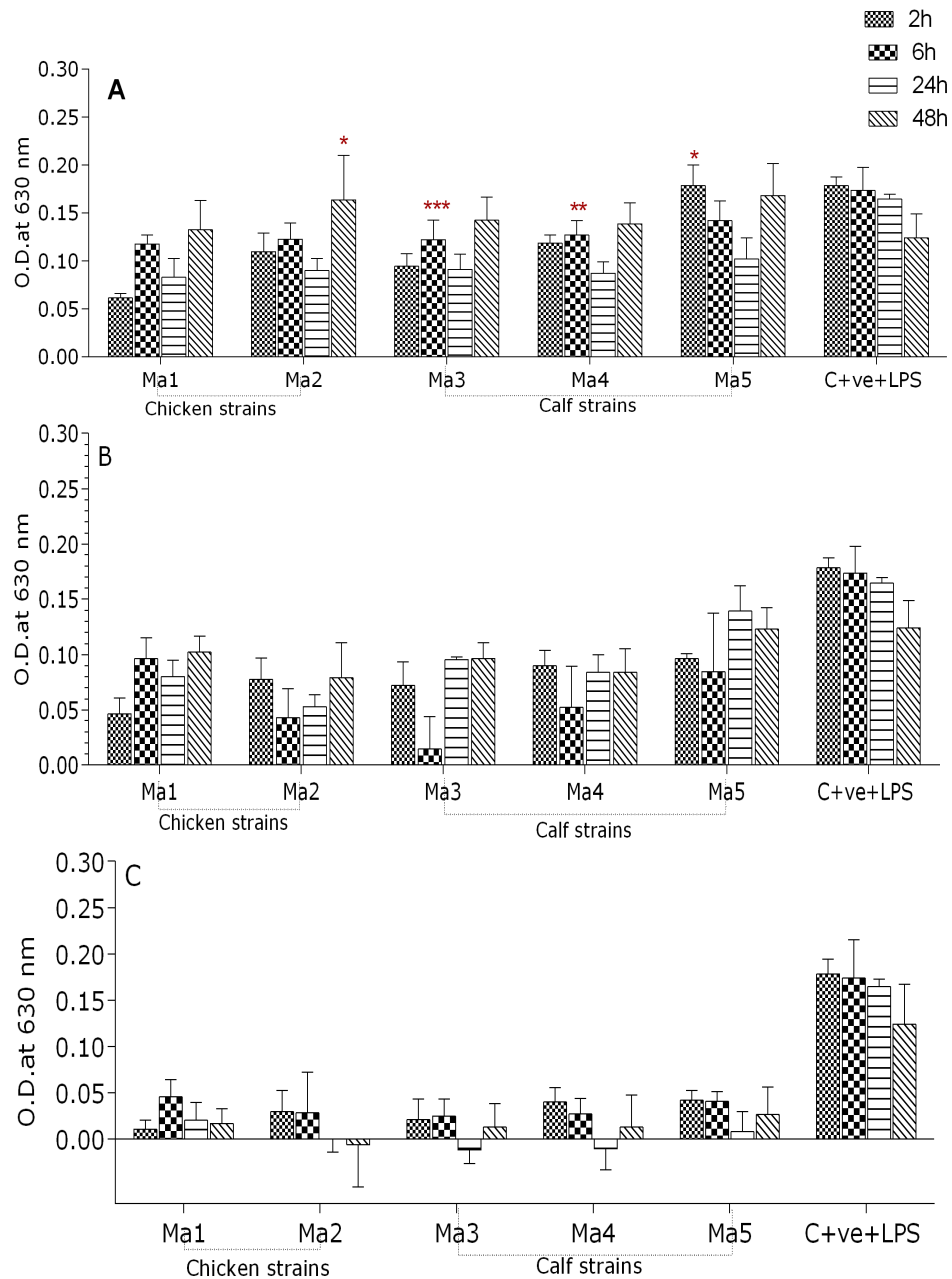


Figure 5.7: Reactive oxygen species production by *M. avium* infected HD11 cells. ROS level is expressed as increase of the optical density at 630 nm (OD 630 nm) above negative control (**A**) Stimulated cells treated with 100 ng/ml of PMA (**B**) Basic group; non-treated HD11 cells. (**C**) Inhibited cells treated with NAC 10mM and DPI 10uM. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08). The values shown are the mean $\pm$ SE of three separate experiments with internal triplicate repeats. Significant differences * p <0.05, ** p <0.01 and *** p <0.001 in the ROS levels changes between time points (2h and 6h, 6h and 24h, 24h and 48h post infection) for each strain.

The intracellular survival of *M. avium* strains Ma1-5 were assessed and determined over 48h post-infection in both THP-1 and HD11 cells in the presence of ROS stimulator and inhibitors and compared with that of non-treated infected cells as control. In the presence of PMA the ROS stimulator the viability of internalised bacteria were significantly higher than that of the controls particularly at 48h post-infection. In contrast when ROS production was inhibited there was a reduction in viability compared to induced and non-treated cells in both THP-1 and HD11 cells (Figure 5.8 and 5.9). There were strain to strain differences in the bacterial sensitivity to the antioxidants, with Ma1 and Ma2 being the most sensitive strains where the numbers of the viable bacteria were greatly reduced at 48h post-infection compared to that of Ma4 and Ma5. These results suggest that antioxidants play a significant role in restricting intracellular survival of the bacterial strains within both THP-1 and HD11 cells. This survival data indicates that *M. avium* grew better in a more oxidizing environment than in the presence of antioxidants; creation of an oxidizing environment by adding PMA to HD11 enhanced *M. avium* growth at 48h post-infection compared to those cells treated with antioxidants $p < 0.05$. This is most clearly seen in HD11 cells where in the presence of the antioxidants, NAC and DPI, growth of Ma2 and Ma4 was completely inhibited at 48h after infection (Figure 5.9). Ma3 was the most tolerant strain to the presence of antioxidants. However, Ma3 showed better growth under oxidizing environments, but no significant differences occurred between PMA-

induced cells and anti-oxidant-treated cells. In general the effects of the antioxidants were more consistent on bacterial survival in HD11 cells than that found in the infected THP-1 cells.

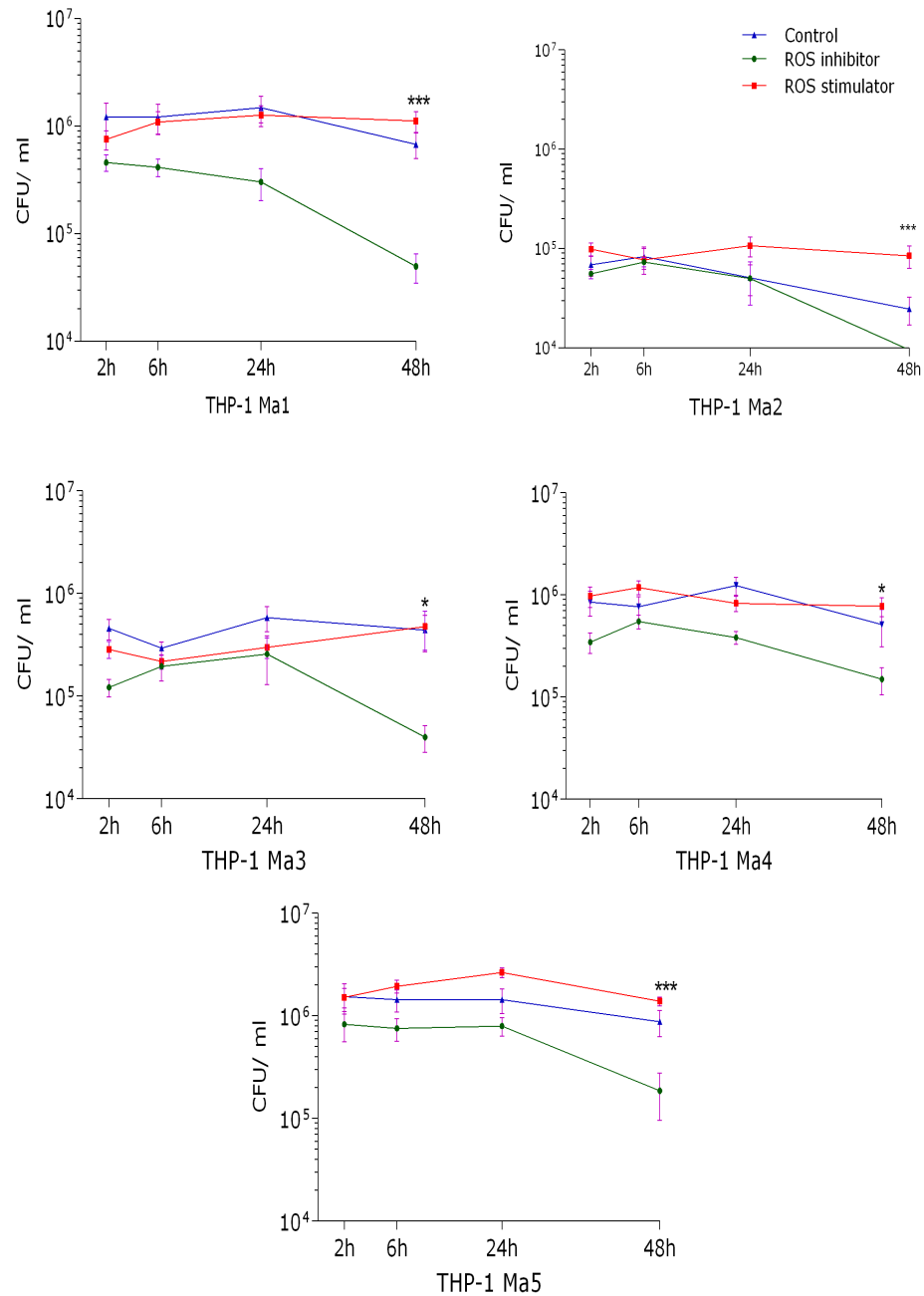


Figure 5.8: Effect of ROS on *M. avium* survival in THP-1 cells. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559.07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08). Samples taken at cells at 2h, 6h, 24h and 48h post infection. (■) Cells treated with 100ng/ml of PMA. (●) HD11 cells treated with NAC 10mM + DPI 10uM (▲) none treated HD11 cells, basic infection. Values shown are means $\pm$ SE of three independent experiments with internal triplicate repeats. * $p < 0.05$, *** $p < 0.001$ - significant differences between survival in the PMA stimulated and inhibitor groups at the each time point.

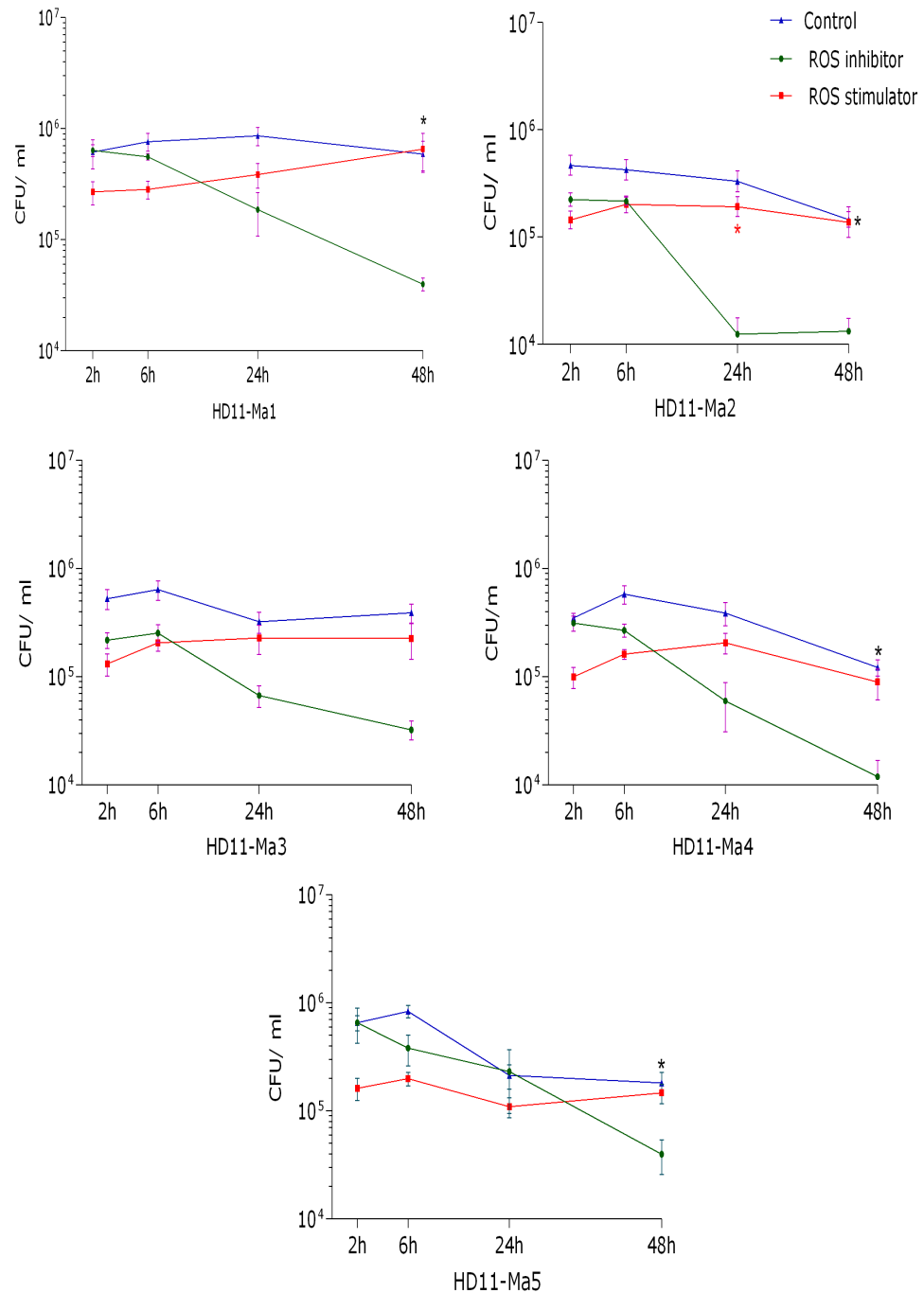


Figure 5.9: Effect of ROS on *M. avium* survival in HD11 cells. Viability of internalised bacteria assessed at 2h, 6h, 24h and 48h. (●) Cells treated with 100n g/ml of PMA. (●) HD11 cells treated with NAC 10mM and DPI 10uM (▲) none treated HD11 cells, basic invasion. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627-08) and Ma5 (61-3728-08). Values shown are mean $\pm$ SE of three independent experiments with internal triplicate repeats. Significance differences $*p < 0.05$ between the ROS stimulated and inhibited cells at the each time point

5.2.2.1. Co-localization of *M. avium* with ROS (gp91^{phox}) in THP-1 and HD11.

To assess the co-localisation of ROS in relation to the survival of the invaded *M. avium* at different time points post-infection, the same procedure used for staining iNOS-bacterial co-localization was used but substituting rabbit-anti-NOX2/gp91^{phox} polyclonal antibody conjugated with Alexa Fluor 555 to stain ROS in both cell lines. This was carried out for strains Ma1 and Ma4. First, ROS location within the cells was detected. (Figure 5.10).

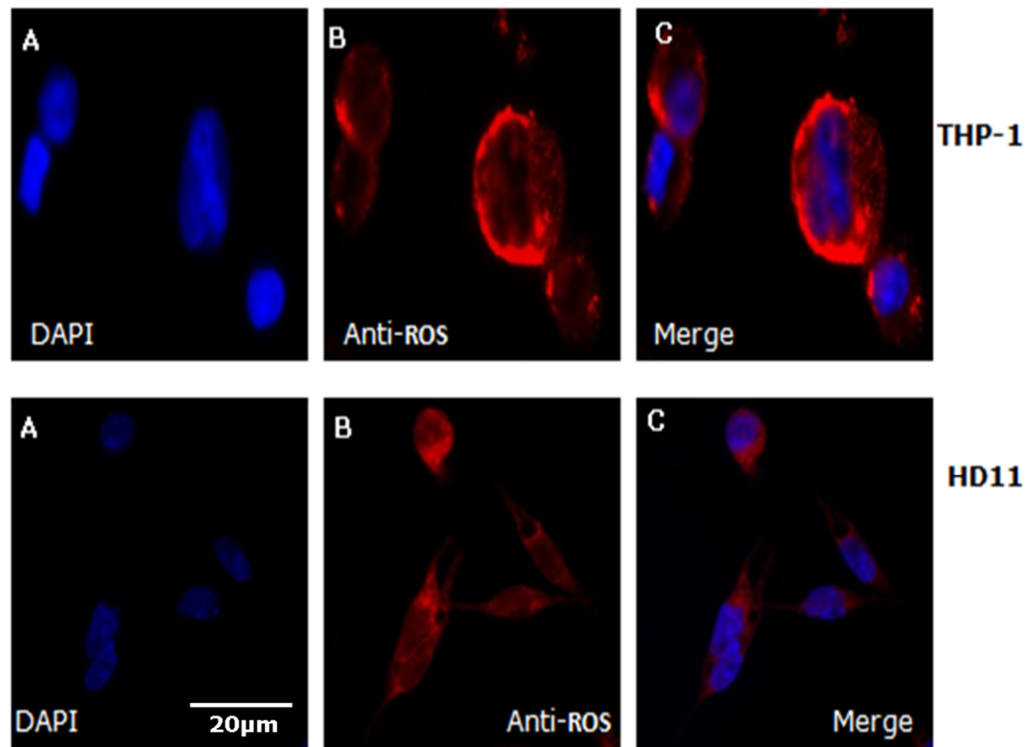


Figure 5.10: Expression of ROS in PMA treated THP-1 and HD11 cells challenged with LPS 50 µg/ ml. Fluorescence microscopic images depict at 6h post-treatment (A) Nuclear DNA visualized by DAPI (4',6-diamidino-2-phenylindole) blue, (B) ROS (gp91^{phox}) red, (C) OVERLAY. Cells were visualized using a LEICA DM 5000B Fluorescent microscope, 63X1.25 lens.

To detect if the presence of ROS is essential for the bacterial growth, a time-course analysis of the co-localized Ma1 (avian isolate) and Ma4 (calf isolate) with NOX2/gp91^{phox} in both cell lines was carried out at different time intervals over 48h. When the bacterial strains were examined for co-localization with NOX2/gp91^{phox} (ROS) by fluorescence microscopy, *M. avium* was visible as a fluorescent green bacillus, the ROS protein showed red and the co-localized bacteria gave a yellow fluorescent colour after merging, while non-co-localized remained green (Figure 5.11).

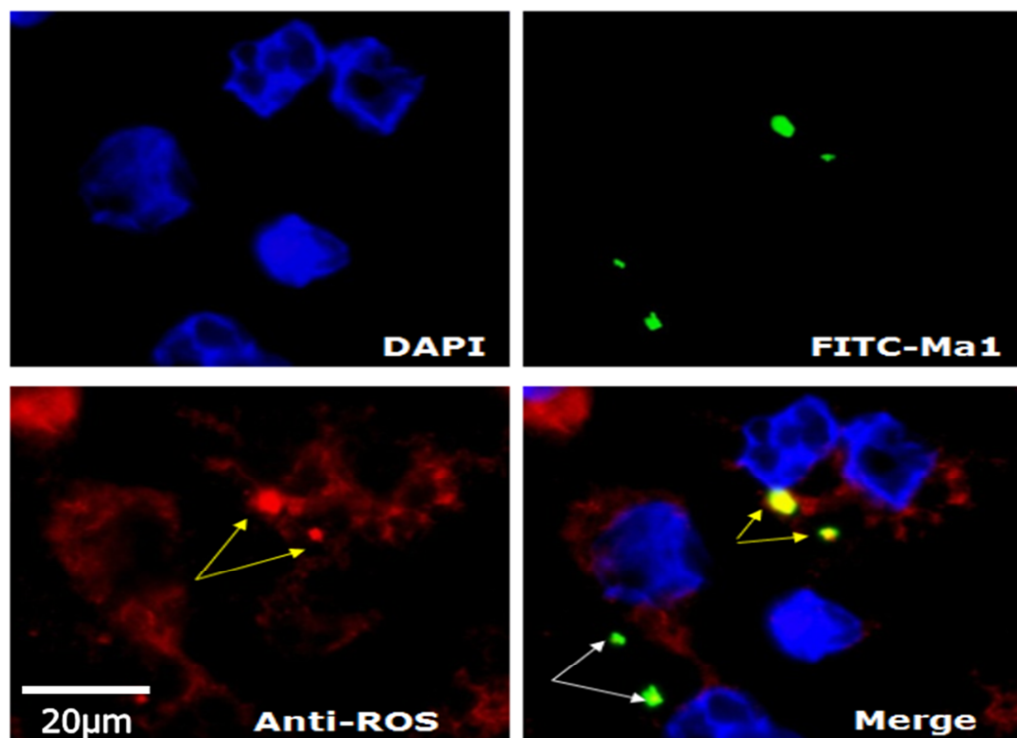


Figure 5.11: Co-localized *M. avium* Ma1 with ROS. Representative fluorescence micrographs of HD11 cells were stained with anti-ROS (gp91^{phox}) conjugated to Alexa 568 (red). Yellow arrows refer to co-localized *M. avium* with ROS (yellow after merged). White arrows refer to green non-co-localized *M. avium* after merging. Scale bar, 20 µm. Cells were visualized using a LEICA DM 5000B Fluorescent microscope, 63X1.25 lens.

In THP-1, the data showed that more than the half of the ingested Ma1 and Ma4 were co-localized with NOX2/gp91^{phox} by 24h post-infection compared to nearly 30% by 6h after infection (Figure 5.12 A, B). A significant correlation was found between the viable numbers of Ma4 and Ma4-ROS-co-localization rate ($p < 0.05$), whereas, the correlation between the viable numbers of Ma1 and Ma1-ROS- co-localization rate was not significant ($p > 0.05$).

In contrast to THP-1, in HD11, the rates of Ma1- and Ma4- ROS-co-localization by 6h post-infection peaked at nearly 50% followed by reduction in the rates over the next time points (Figure 5.12 C and D). There were significant positive correlations between the viable numbers of Ma1- and Ma4 -ROS-co-localization rates in infected HD11 cells ($p < 0.05$).

The results indicate that the increased co-localization rates of the bacteria with ROS (NOX2/gp91^{phox}) correlated with the increased in the numbers of the viable bacteria of the bacterial strains in both cell lines at different time points post-infection. The data suggested that ROS plays an important role in maintaining bacterial growth.

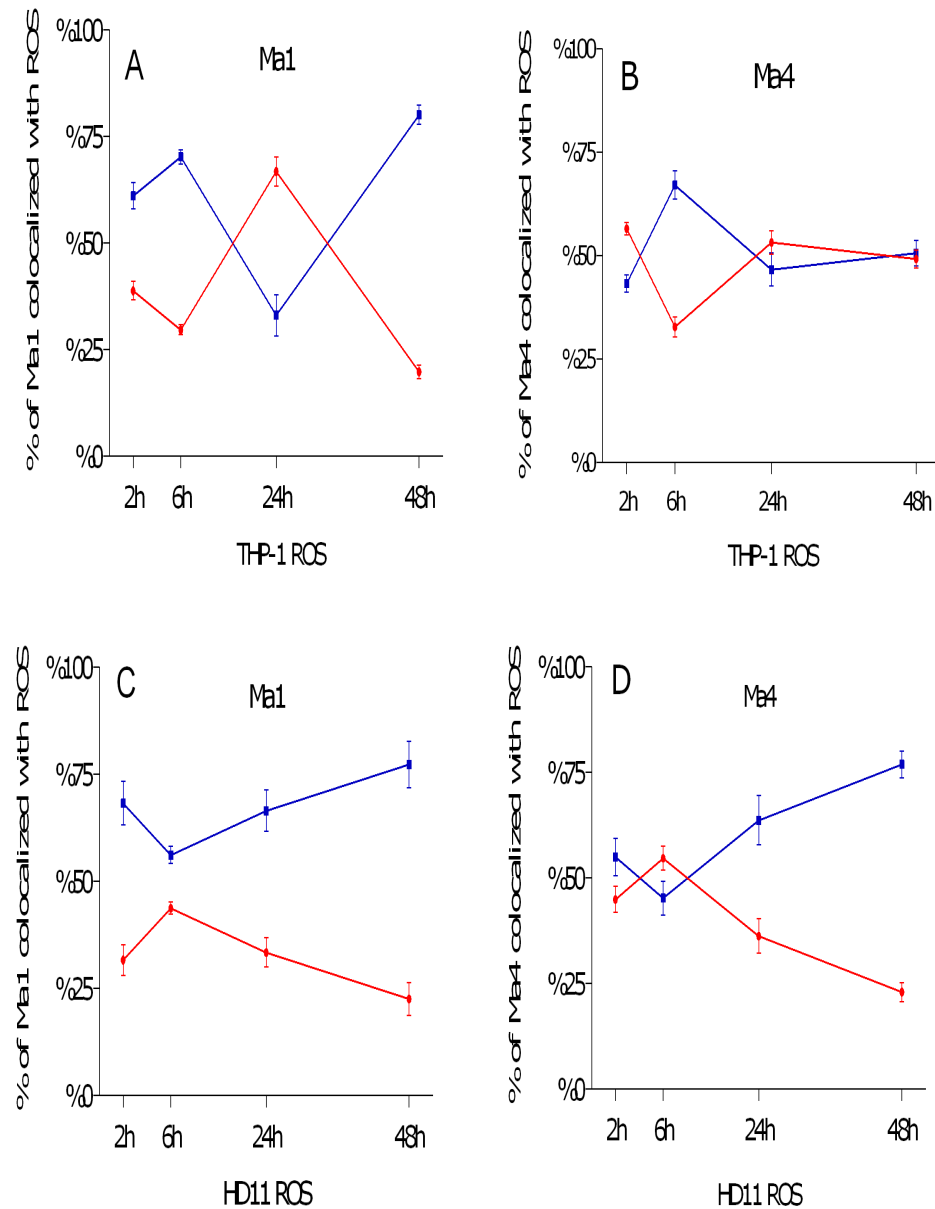


Figure 5.12: Quantitative analysis of ROS co-localisation with *M. avium* Ma1 and Ma4 in THP-1 and HD11. (A) Ma1 in THP-1 (B) Ma4 in THP-1 (C) Ma1 in HD11 and (D) Ma4 in HD11. Co-localised mycobacteria with ROS were counted over different time points in infected cells. (■) colour refers to the co-localised *M. avium* with ROS (■) refers to non-co-localised *M. avium*. The percentage of co-localised and non-co-localised bacteria are shown as mean $\pm$ SD at each time point in 50 microscopic fields of duplicate cover slips of two separated experiments

5.3. Discussion

The ingested mycobacteria are exposed to different antimicrobial agents within infected macrophages. Reactive oxygen species and nitric oxide are crucial intracellular killing mechanisms of macrophages and other phagocytic cells. Nitric oxide has been widely reported to play a vital role in intracellular survival of *M. tuberculosis* and *M. bovis* in murine macrophages, while in human macrophages the role of nitric oxide is not well understood (Nozaki et al., 1997). Many studies have been carried out on the interaction between these innate killing mechanisms and mycobacteria in mammalian cells (Rhoades and Orme, 1997, O'Brien et al., 1994, Pozos and Ramakrishan, 2004, Yang et al., 2009, Fenton et al., 2005, Shin et al., 2008) but nothing has been reported about *M. avium* in chicken cells. Therefore this study was carried out to better understand the role of innate ROS and nitric oxide during *M. avium* infection of avian macrophages. In this study we showed a strong correlation between the increases in nitric oxide production and the decreases in the numbers of viable bacteria recovered from infected HD11 over 48h post-infection. The levels of induced nitric oxide produced vary between bacterial strains which might be associated with the virulence of the bacteria, some strains were found to induce more nitric oxide than others. Ma1 and Ma3 induced lower levels of nitric oxide compared to that produced by the other strains, while the numbers of viable bacteria of Ma1 and Ma3 were higher than the other strains (Ma4, Ma5 and Ma2) at 48h post-infection. The role of

nitric oxide in the bacterial survival was further verified by using a nitric oxide stimulator and inhibitors and also by calculating the percentages of the bacteria co-localized with iNOS over 48h post-infection. The data showed that the intracellular growth of *M. avium* strains was significantly suppressed at higher levels of nitric oxide. These data are in line with published data on the killing of *M. bovis* infection in murine J774 macrophages (Anes et al., 2006).

In contrast to HD11 cells, THP-1 cells are unable to produce nitric oxide, but the bacterial numbers decreased at 48h post-infection. Nathan and Shiloh (2000) reported that the bactericidal role of nitric oxide in *M. tuberculosis* infection of human macrophages is not important for suppression of the bacterial growth in early infection. This suggests a nitric oxide independent killing mechanism must also exist.

Despite the fact that it is widely accepted that the ROS enhances the ability of the infected cell to control the intracellular survival of the ingested bacteria, ROS has been seen to assist the growth of *M. tuberculosis* and *M. abscessus* in infected mammalian macrophages (Oberley-Deegan et al., 2010b, Oberley-Deegan et al., 2009, Oberley-Deegan et al., 2010a). This is confirmed by the data presented here which shows that intracellular survival of all the *M. avium* strains tested was enhanced in an oxidizing environment compared to less oxidising conditions in both cell types.

While an oxidising environment is important in survival of mycobacteria the interactions between ROS and nitric oxide are also considered to be essential for killing these intracellular bacteria. Superoxide and nitric oxide are showed to react and form a very toxic component known as Peroxynitrite (ONOO⁻) (Deffert et al., 2014) and the antioxidant glutathione acts as a carrier molecule for nitric oxide in the form of S-nitrosogluthathione, glutathione and S-nitrosogluthathione which are bactericidal (Deneke and Fanburg, 1989, Jagannath *et al.*, 1998, Morris and Hansen, 1981). Thus, the present data indicate that both nitric oxide and antioxidants and the interaction between them play important roles in bacterial survival.

The intracellular location of iNOS relative to *M. avium* may also be critical for its killing effects. The co-localisation data showed that the delivery of iNOS to the phagosomes is strain dependent. Co-localisation of iNOS was faster for Ma4 than Ma1 and the overall percentage of the co-localization with Ma1 was lower than that of Ma4 during the first 24h post-infection. This suggests that Ma1 may to some extent prevent delivery of iNOS to the phagosomes. Interestingly Ma1 caused condensation and aggregation of the cell tubulin in a similar way as the cells which were treated with tubulin depolymerising agent nocodazole, while Ma4 was unable to induce depolymerisation of the cell cytoskeleton (Seen next chapter Figure 6.4). It has previously been reported that iNOS is anchored to the cell cytoskeleton of murine macrophages and that an intact cell cytoskeleton is essential to deliver iNOS to the phagosomes

containing microorganisms (Defacque et al., 2000, Miller et al., 2004).

The quantitative co-localization of ROS with Ma1 and Ma4 in both cell lines was also determined. There were host cell differences; in THP-1 the peak levels of the co-localized bacteria were found at 24h post-infection, in contrast to that found at 6h after infection in HD11 cells. The difference between the cells used may be due to the rapidity of ROS production. ROS production in chicken macrophages is faster than in mammalian cells (Hamilton and Adams, 1987). For controlling bacterial growth, it would be important to understand what factors are involved in the exclusion of iNOS and ROS from the bacterial phagosomes at 48h post-infection in both cell lines.

It can be concluded that innate antimicrobial mechanisms play important roles in limiting intracellular survival of *M. avium* in both cells lines. There were differences in the role of innate immune responses between THP-1 and HD11 cells with chicken cells killing the intracellular bacteria via nitric oxide. In contrast to usual role of ROS in controlling the many other intracellular pathogens, in this study it has been seen that *M. avium* grew well in an oxidizing environment. This is in line with observations in mammalian studies with *M. bovis*. Moreover, the antioxidants were very effective in limiting the bacterial survival in the absence or the presence of nitric oxide. While not definitive proof the data presented in this report

supports a similar process occurs in both avian and human cells during *M. avium* infection.

6.0 Survival mechanisms of *M. avium* in host cells.

6.1 Introduction

Despite the fact that macrophages are very effective in phagocytosis and killing most invading bacteria, pathogenic mycobacteria, such as *M. tuberculosis*, are known to survive and replicate within infected mammalian cells (Ernst, 1998). Normally, the ingested particles within the macrophages are delivered into the acidic environment of the phagolysosomes for lysis and degradation (Xu et al., 1994). The ability of mycobacteria to replicate in the host macrophage is critical to its pathogenesis, emphasizing a need for a clearer understanding of its interactions with the host macrophage (Sly et al., 2003). However, the survival mechanisms of *M. tuberculosis* and *M. bovis* BCG within mammalian cells are not fully understood. It has been suggested, based on the results of mammalian infection studies, that the bacteria circumvent the intracellular hostile environment of infected cells via several different strategies (Ernst, 1998), including: (i) entry through a specific receptor (Tessema et al., 2001b) (ii) disruption of the host cell cytoskeleton (Ferrari et al., 1999); (iii) release of the bacteria from the phagocytic vacuoles to the cell cytosol (Stamm et al., 2003); (iv) inhibition of phagosome acidification (Meena, 2010); and (v) interfering with phagolysosome maturation (Vergne et al., 2003).

It is unknown whether *M. avium* uses the same mechanisms to survive in avian cells. The work presented in this chapter was carried out to understand the role of receptors and cytoskeleton in intracellular survival of *M. avium* and also to better understand the survival strategies used by the bacteria in avian cells

6.2 Results

6.2.1 Effects of individual receptor on intracellular survival of *M. avium* in the host cells.

To determine if the invasion of THP-1 and HD11 cells via specific receptors could have any effect on the fate of the ingested bacteria, the intracellular survival of the bacterial strains were determined in both cell lines in the presence of competitive receptor blockers. The numbers of the viable bacteria were examined at different time points post-infection over 48h.

In THP-1 cells, the role of the individual receptor blockage on bacterial survival was different depending on the bacterial strain involved. At 6h post-infection, the bacterial survival within the treated cells with the receptors blockers was significantly ($p < 0.05$) reduced compared to the control, except for Ma3 in CR3 blocked cells. In THP-1 cells blocked for scavenger receptors, survival of Ma1 and Ma2 continued to decrease over the 48h, whereas the kinetics of Ma3, Ma4 and Ma5 were increased by 24h and 48h post-infection in contrast to that of the control group where the bacterial survival was sharply reduced by 48h post infection. Cells blocked for mannose receptors also showed variation in the survival between the bacterial strains when compared to the control group of non-treated infected cells. The kinetics of the intracellular survival of Ma3 and Ma4 in mannose receptor-blocked THP-1 cells were similar to that found in non-treated THP-1 cells (control group) at 24h post-

infection whereas the viable numbers of Ma2 and Ma3 continue to increase by 48h after infection. Viable Ma1 and Ma5 bacteria did not increase during the experiment. By blocking complement receptor CR3, THP-1 cells were also unable to limit the bacterial growth of Ma2, Ma4 and Ma5 particularly at 24h post-infection, whereas the cells were efficient in restricting growth of strains Ma1 and Ma3 (Figure 6.1).

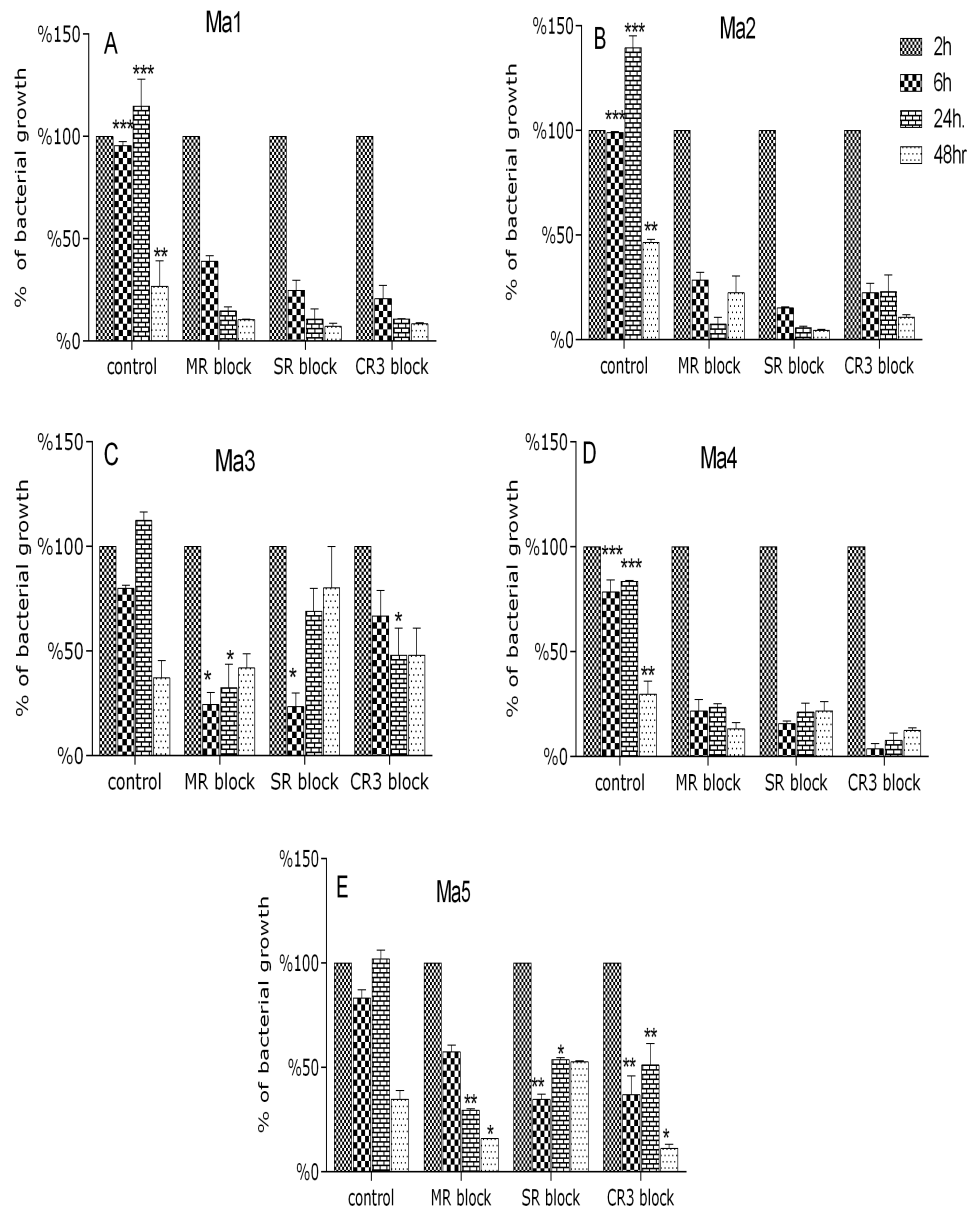


Figure 6.1: Effect of receptor-mediated entry on survival *M. avium* in THP-1 cells. Intracellular counts were determined at 2h, 6h, 24h and 48h and are represented as percentage of initial invasion at 2h post-infection. Strains **(A)** *M. avium* Ma1 (NC08562.02), **(B)** Ma2 (NC08559-07), **(C)** Ma3 (61-3623-05), **(D)** Ma4 (61-4627-08) and **(E)** Ma5 (61-3728-08). Heparin sodium salt from porcine intestinal mucosa 10 U/ml was used for blocking complement receptors CR3. Dextran sulfate sodium salt 100 ug/ml for blocking scavenger receptors (SRs). α -Methyl *D*-Mannoside 2% was used for mannose receptors (MRs). The values shown are the means for three separate experiments with internal triplicate repeats. ** $p < 0.01$ and *** $p < 0.001$, indicate significance differences between treated and non-treated cells at each time point post infection reported by each mycobacterial strain.

In HD11 cells, the intracellular survival of the bacteria greatly depended on the invasion mechanism. Scavenger-, mannose- and complement CR3 receptor-blocked HD11 cells had reduced bacterial survival compared to the control group, except for Ma3 at 24h post-infection in scavenger receptor-blocked cells (Figure 6.2).

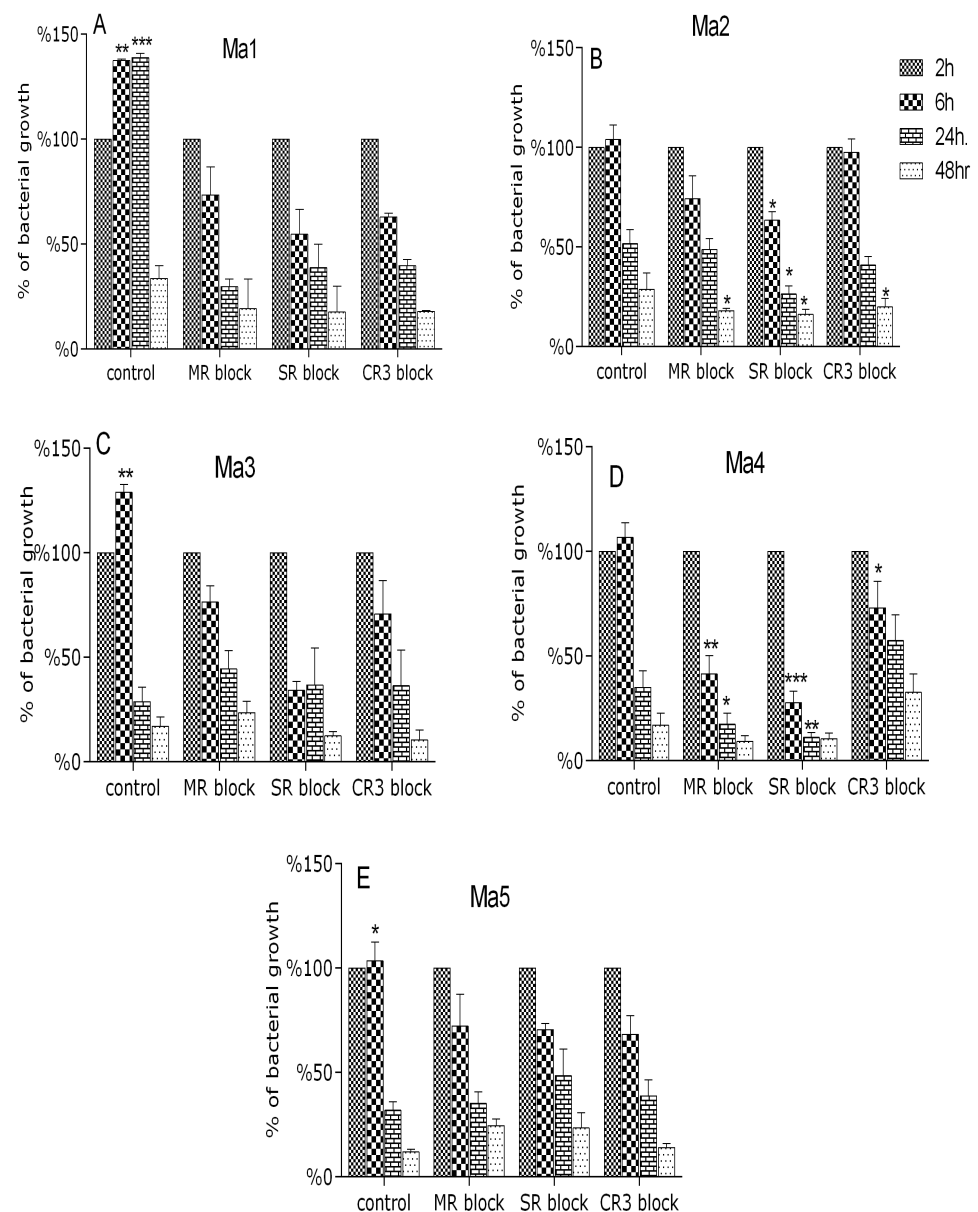


Figure 6.2: Effect of receptor-mediated entry on survival *M. avium* in HD11 cells. Intracellular counts were determined at 2h, 6h, 24h and 48h and are represented as percentage of initial invasion at 2h post-infection. Strains **(A)** *M. avium* Ma1 (NC08562.02), **(B)** Ma2 (NC08559-07), **(C)** Ma3 (61-3623-05), **(D)** Ma4 (61-4627-08) and **(E)** Ma5 (61-3728-08). Heparin sodium salt from porcine intestinal mucosa 10 U/ml was used for blocking complement receptors CR3. Dextran sulfate sodium salt 100 ug/ml used for blocking scavenger receptors (SRs). α -Methyl *D*-Mannoside 2% was used for mannose receptors (MRs). The values shown are the means for three separate experiments with internal triplicate repeats. * $p < 0.5$, ** $p < 0.01$ and *** $p < 0.001$, indicate significance differences between treated and non-treated cells at each time point post infection reported by each mycobacterial strain.

6.2.2 Localization of *M. avium* within HD11 cell compartment

To determine the role of the intracellular location of *M. avium* in the bacterial survival at different time points after infection, and to understand the intracellular trafficking of the five *M. avium* strains within the chicken cells, immunofluorescent and Transmission Electron Microscopy techniques were used. Photographs of actin and tubulin in *M. avium* Ma1-5 infected HD11 cells were taken and compared with controls.

Infection with *M. avium* had no obvious impact on the actin distribution in HD11 cells when compared to mock infected and cytochalasin D treated cells (Figure 6.3).

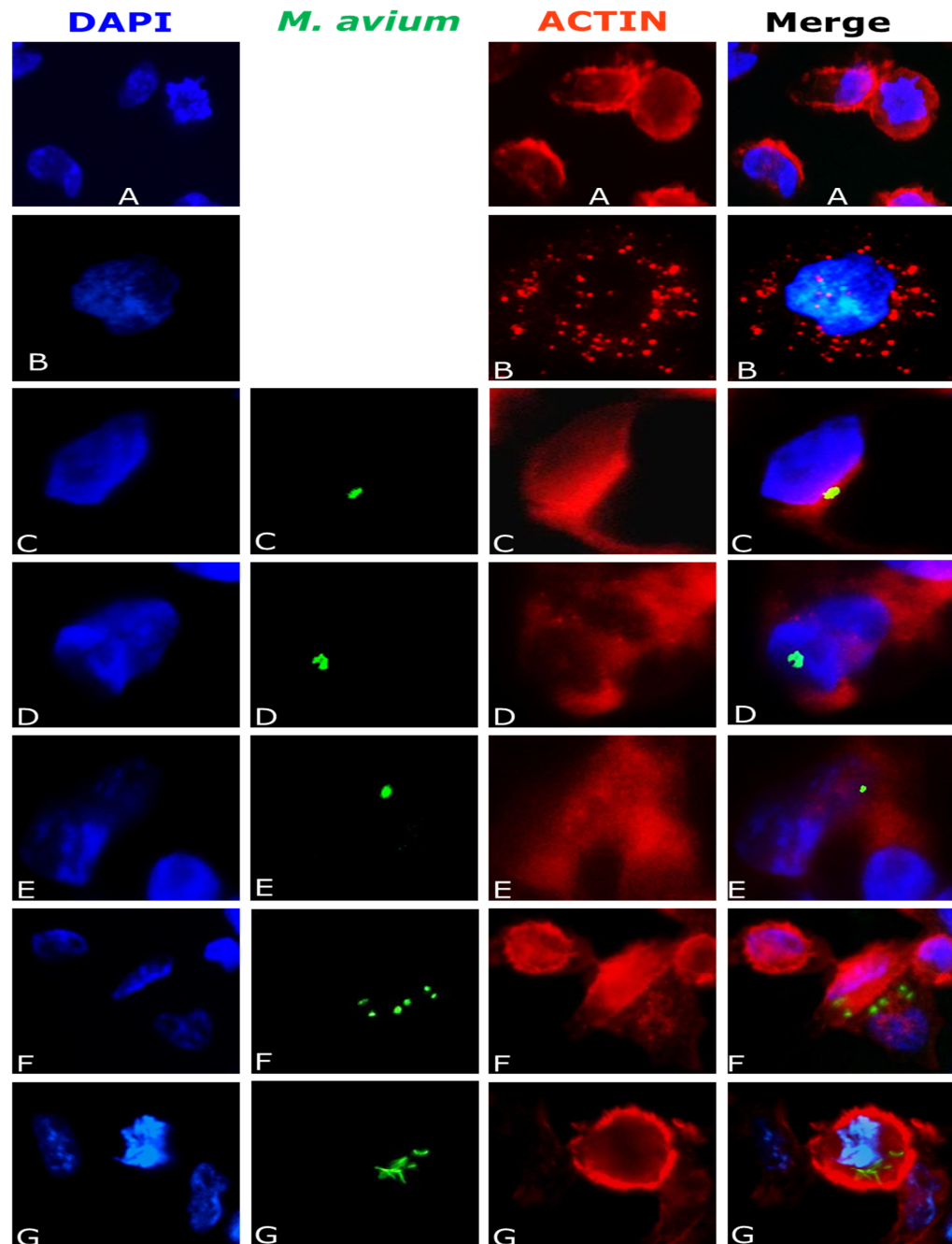


Figure 6.3: A representative image shows actin morphology in *M. avium* infected HD11 cells. Images taken at 24h post-infection. Row (A) Non-treated, (B) Cytochalasin D treated cells at 1 μ g/ ml (C) Ma1- (D) Ma2- (E) Ma3- (F) Ma4- (G) Ma5-infected cells. Columns as indicated. DNA is stained with DAPI (blue). *M. avium* stained with green fluorescence Auramine O-rhodamine (green). Actin stained with Alexa Fluor™ 546 Phalloidin (red) and The actin morphology was detected by an immunofluorescence microscope. Scale bar 20 μ m.

Immunofluorescence was used to study the tubulin distribution in *M. avium* infected HD11 cells. Strain to strain differences could be observed with Ma1, Ma2 and Ma3 showing disruption of the tubulin similar to that observed of the nocodazole control. Ma4 and Ma5 had intact tubulin equivalent to that observed in the mock-infected cells (Figures 6.4)

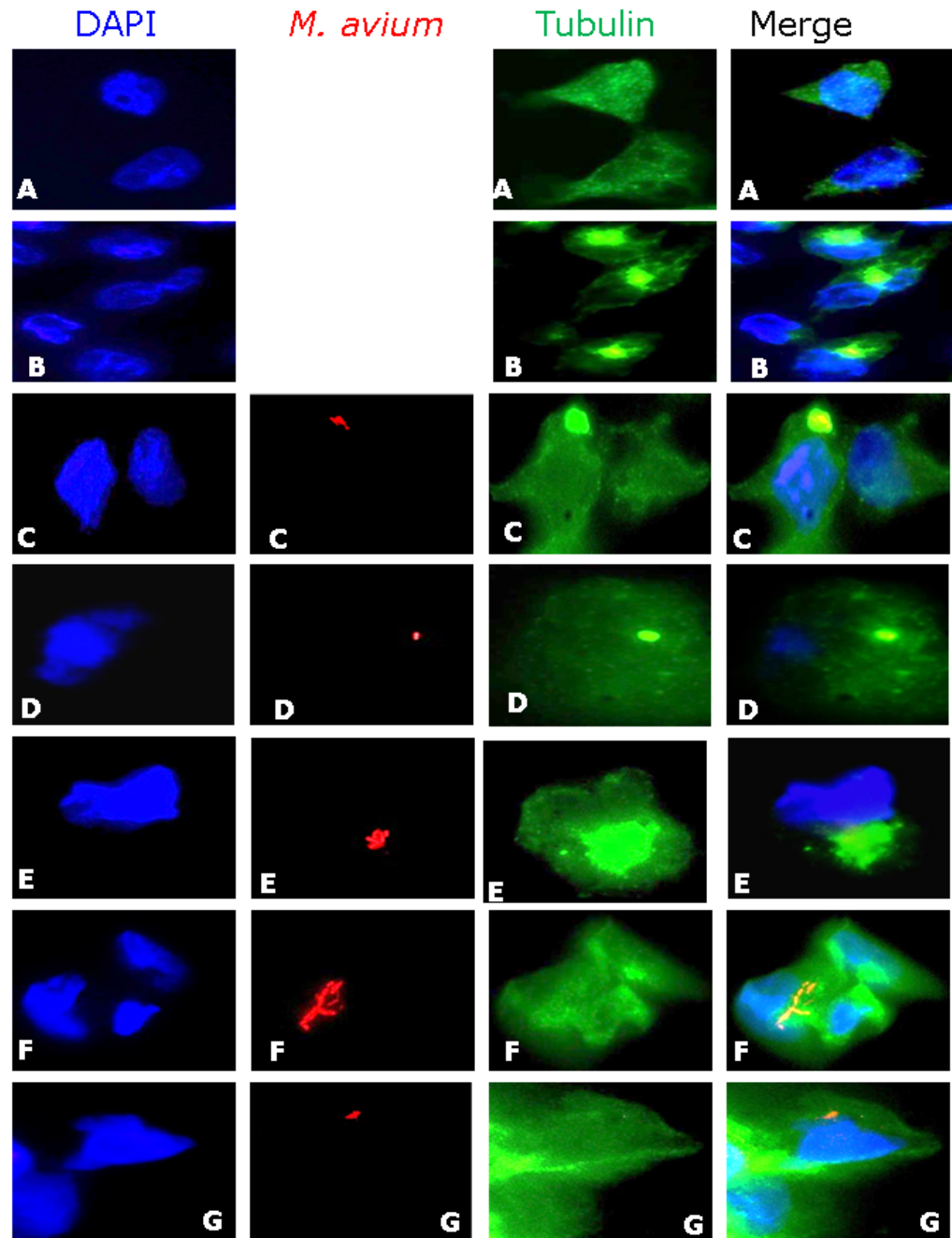


Figure 6.4: Tubulin morphology in *M. avium* infected HD11 cells. Row (A) mock-infected cell, (B) Nocodazole treated cells at 20uM, (C) Ma1- (D) Ma2- (E) Ma3- (F) Ma4- (G) Ma5-infected cells. Columns as indicated. DNA is stained with DAPI (blue). *M. avium* (red) with goat anti-mouse IgG1 anti-*M. avium* monoclonal antibodies gamma chain (Cy5®) and tubulin is stained with goat anti rabbit IgG H&L (FITC) against rabbit anti-chicken tubulin polyclonal antibody (green). Images taken at 24 hours post-infection. Scale bar 20 μ m.

Other studies have indicated post-invasion that the disruption of cell tubulin was associated with the release of *Shigella* from the phagocytic vacuoles to the cell cytosol (Yoshida et al., 2006). To confirm if this was associated with *M. avium* strains Transmission Electron Microscopy was used. This showed that the bacterial strains were visible exclusively within phagosomes (Figure 6.5). These findings suggest that the bacterial strains manipulate the cell tubulin in a way to hinder the phagosomal maturation and make the phagocytic vacuoles suitable niches for their survival and growth.



Figure 6.5: Representative TEM micrograph of HD11 cells infected with *M. avium*. Cells were processed at 6h post-infection. *M. avium* are found inside phagosomes (single bacterium (n=127) per a vacuole). Scale bar is 10000nm. Labels N= nucleus, P= phagosome and M= *M. avium*.

6.2.3 The role of cytoskeleton in intracellular survival of *M. avium* in the host cells.

The effect of actin and tubulin disruption on bacterial survival in THP-1 and HD11 cells was determined. The intracellular survival data of the bacterial strains showed strain to strain variation in the numbers of intracellular survival of the viable bacteria within the treated cells over 48h (Figure 6.6). In THP-1 cells treated with inhibitors cytochalasin D and nocodazole, survival was generally reduced over 24 hours compared to untreated cells. In nocodazole treated cells at 48h post-infection, there were increases in bacterial numbers except with Ma5, whereas, in the presence of cytochalasin cells the numbers of the viable bacteria were decreased during infection except Ma3 at 48h and Ma4 at 6h post-infection.

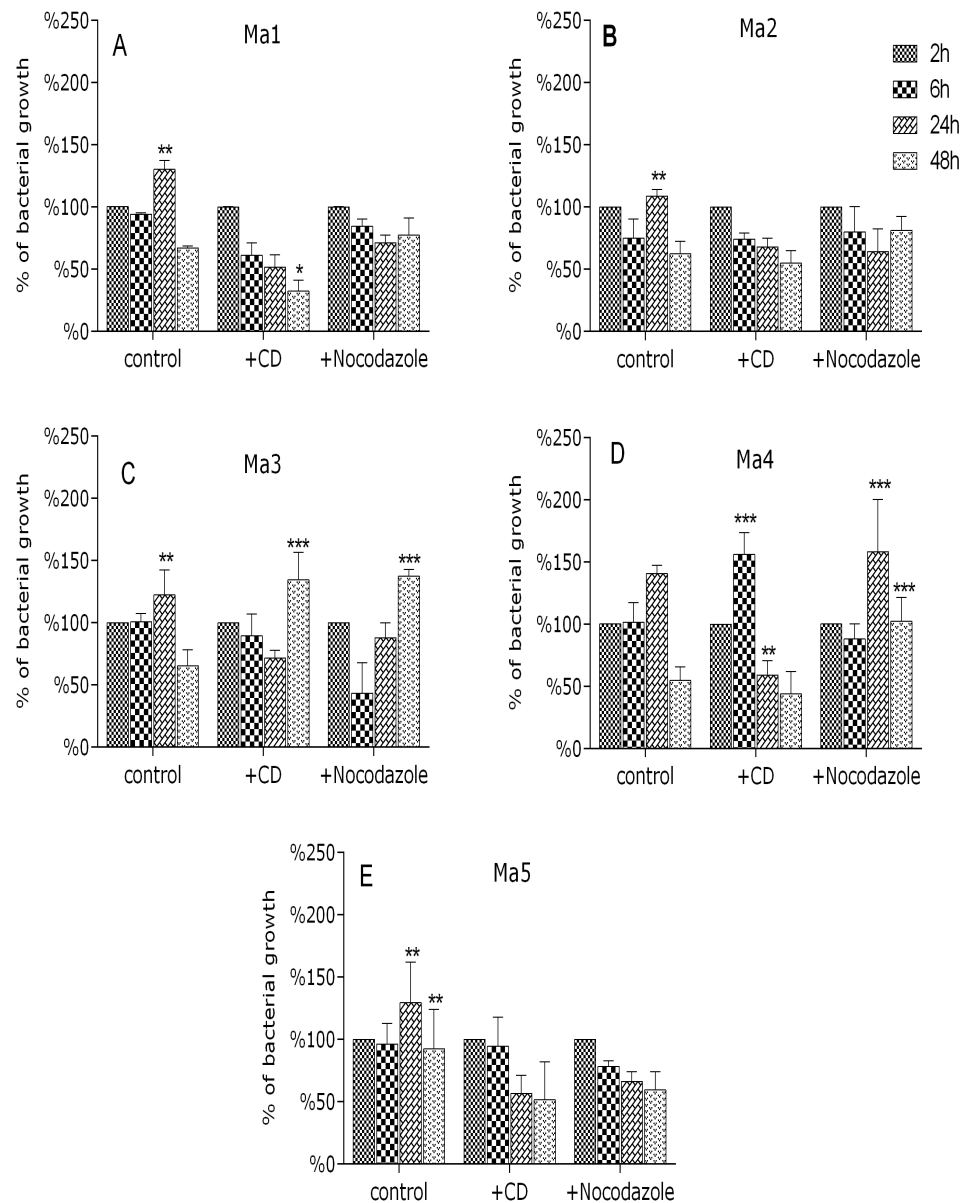


Figure 6.6: Effect of actin and tubulin on survival *M. avium* in THP-1 cells. Intracellular counts were determined at 2h, 6h, 24h and 48h and are represented as percentage of initial invasion at 2h post-infection. Strains **(A)** *M. avium* Ma1 (NC08562.02), **(B)** Ma2 (NC08559-07), **(C)** Ma3 (61-3623-05), **(D)** Ma4 (61-4627.08) and **(E)** Ma5 (61-3728.08). Cytochalasin D (CD) at 1 μ g/ml was used for actin disruption and nocodazole (Noco) at 20 μ M used to disrupt tubulin. The values shown are the means for three separate experiments with internal triplicate repeats. ** $p < 0.01$ and *** $p < 0.001$, indicate significance differences between treated and non-treated cells at each time point post-infection reported by each mycobacterial strain.

The intracellular survival of the bacterial strains *M. avium* Ma1-5 in HD11 cells was also determined in the presence of cytoskeleton inhibitors. The bacterial viable numbers in cytochalasin D-treated cells were reduced at 6h post-infection but increased by 24h post-infection (Figure 6.7) compared to untreated cells with the exception of Ma3. In nocodazole-treated cells, the survival of the bacteria were increased at 6h post infection and then followed by sharp reductions over next time points 24h and 48h post-infection.

In both cell lines, the intact cytoskeleton was found to play a role in bacterial survival. However, there were host cell and strain dependent differences. The effects of actin and tubulin on bacterial growth were different between the cells used and between the bacterial strains.

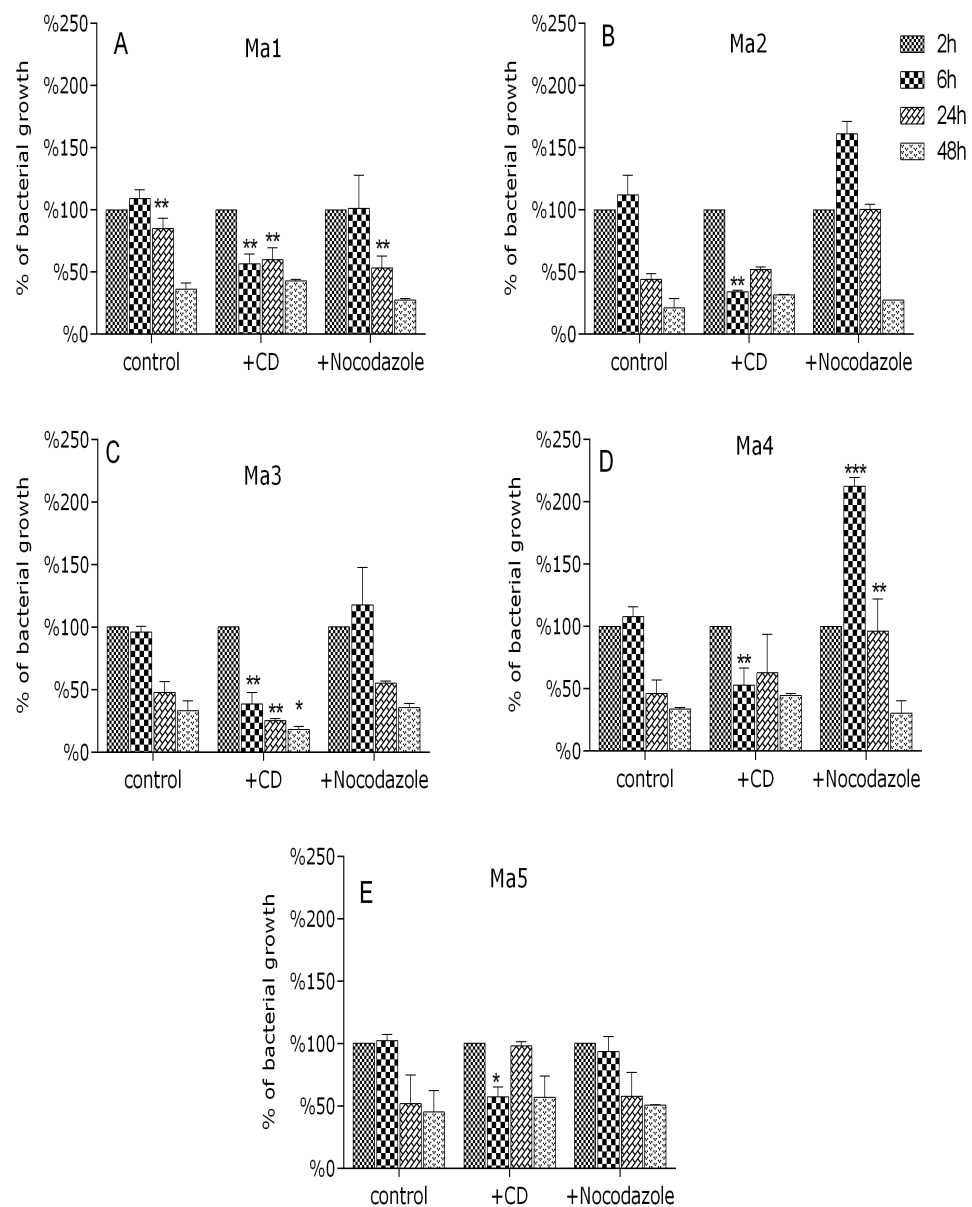


Figure 6.7: Effect of actin and tubulin on survival *M. avium* in HD11 cells. Intracellular counts were determined at 2h, 6h, 24h and 48h and are represented as percentage of initial invasion at 2h post-infection. Strains **(A)** *M. avium* Ma1 (NC08562.02), **(B)** Ma2 (NC08559-07), **(C)** Ma3 (61-3623-05), **(D)** Ma4 (61-4627.08) and **(E)** Ma5 (61-3728.08). Cytochalasin D (CD) at 1 μ g/ml was used for actin disruption and nocodazole (Noco) at 20 μ M used to disrupt tubulin. The values shown are the means for three separate experiments with internal triplicate repeats. * p <0.05, ** p <0.01 and *** p <0.001, indicate significance differences between treated and non-treated cells at each time point post infection reported by each mycobacterial strain.

It was suggested that differences in survival may relate to a reduction in nitric oxide due to disruption of the cytoskeleton by infecting bacteria. To test whether disruption of cytoskeleton components can reduce nitric oxide, HD11 cells were treated with either with cytochalasin D or nocodazole and assessed for LPS induced nitric oxide production. Cells were challenged with LPS 50 $\mu\text{g/ml}$ (final concentration) in the presence of cytochalasin D and nocodazole. The data indicated that both disrupted actin and tubulin reduced LPS induced nitric oxide production compared with the control positive values ($p < 0.01$) (Figure 6.8).

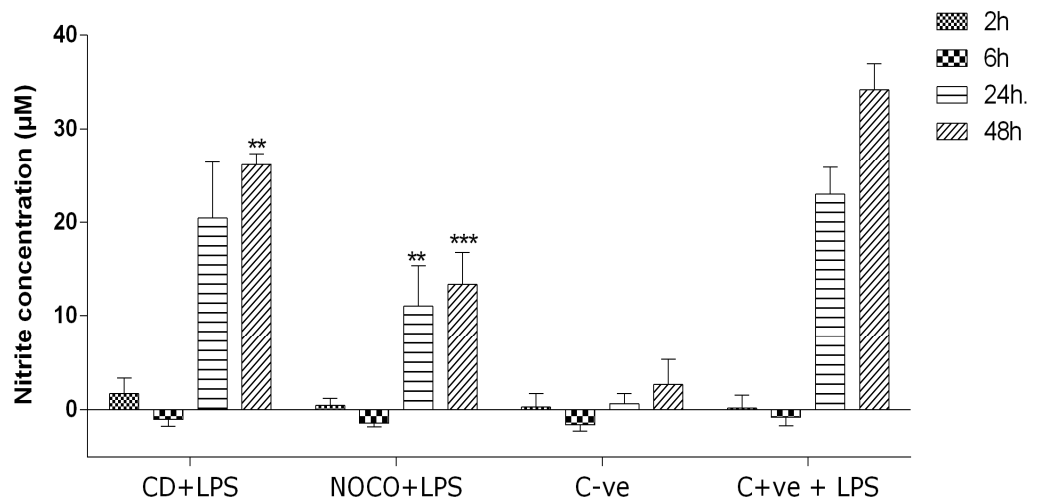


Figure 6.8: Effect of cytoskeleton disruption on nitrite production by LPS treated HD11 cells over 48h. Values shown are the mean $\pm$ SE of three independent experiments with internal triplicate repeats. ** $p < 0.01$ and *** $p < 0.001$ indicate the differences between the LPS 50 $\mu\text{g/ml}$ positive control group and CD 1 $\mu\text{g/ml}$ and nocodazole 20 μM control groups at each time point.

As well as reducing overall production of nitric oxide localisation with bacteria may also be affected by cytoskeletal disruption. To follow the effects of tubulin disruption on nitric oxide distribution during

infection of HD11 cells immunostained iNOS (red) was co-localized with FITC-stained tubulin (Figure 6.9). The merged images of iNOS and tubulin indicate co-localization (in yellow) throughout the cells. Treatment with nocodazole changed the intracellular distribution of iNOS and tubulin, which appeared aggregated in clumps, and condensed toward the centre of the treated cells. In the absence of nocodazole, iNOS staining showed cytoplasmic staining which was diffuse and evenly distributed in all cells. Given that some strains disrupt tubulin and we have shown tubulin disruption can reduce nitric oxide we hypothesise that this disruption of tubulin by Ma1-, Ma2- and Ma3-infected HD11 cells may be associated with low nitric oxide production and increased survival properties.

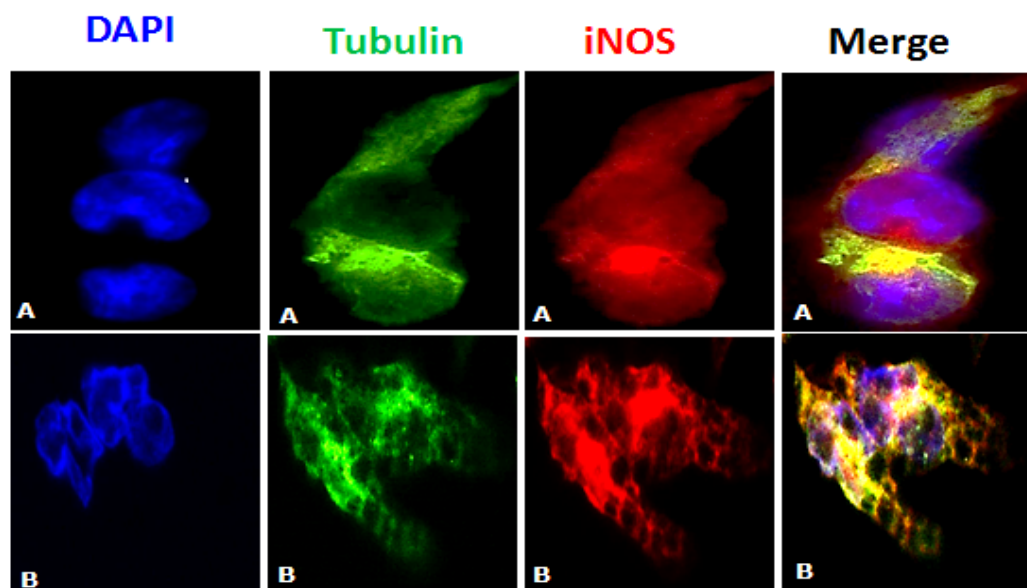


Figure 6.9: Disruption of co-localisation iNOS with tubulin by nocodazole in HD11 cells. Row (A) mock-infected cell and (B) Nocodazole treated cells at 20uM. Tubulin is stained with goat anti rabbit IgG H&L (FITC) against rabbit anti-chicken tubulin (green) and DNA with DAPI (blue). iNOS (red) with goat anti-rabbit IgG anti-chicken iNOS. Scale bar 20 µm.

6.2.4 Phagosomal pH of *M. avium* in infected HD11 cells

It appears that the bacteria may be disrupting the maturation of the phagosome. This may change the environment around the bacteria leading to improved survival. To test the effect of mycobacterial infection on phagosomal pH in infected HD11 cell CF/Rho emission ratio method was used to determine the phagosomal pH during infection.

At 2h post-infection the pH values in the phagosomes containing five *M. avium* strains were between pH 6 and pH 6.5 which were not-significantly different from the Zymosan A control. The pH for Ma3 was significantly higher than the Zymosan A control ($p < 0.05$). At 6h post-infection the pH of phagosomes containing all five mycobacterial strains and Zymosan A were further reduced compared to previous 2h time point. However, Ma1, Ma2 and Ma3 were able to maintain their environmental pH values at greater than 5.5 compared to the values for Ma4, Ma5 and Zymosan A which were reduced to less than pH5 ($p < 0.01$) (Figure 6.10). By 24h and 48h post-infection the values of pH in vacuoles containing all five *M. avium* strains varied between 4.5 and 5.5 which was significantly higher than that of Zymosan A treated cells at pH-4 ($p < 0.01$).

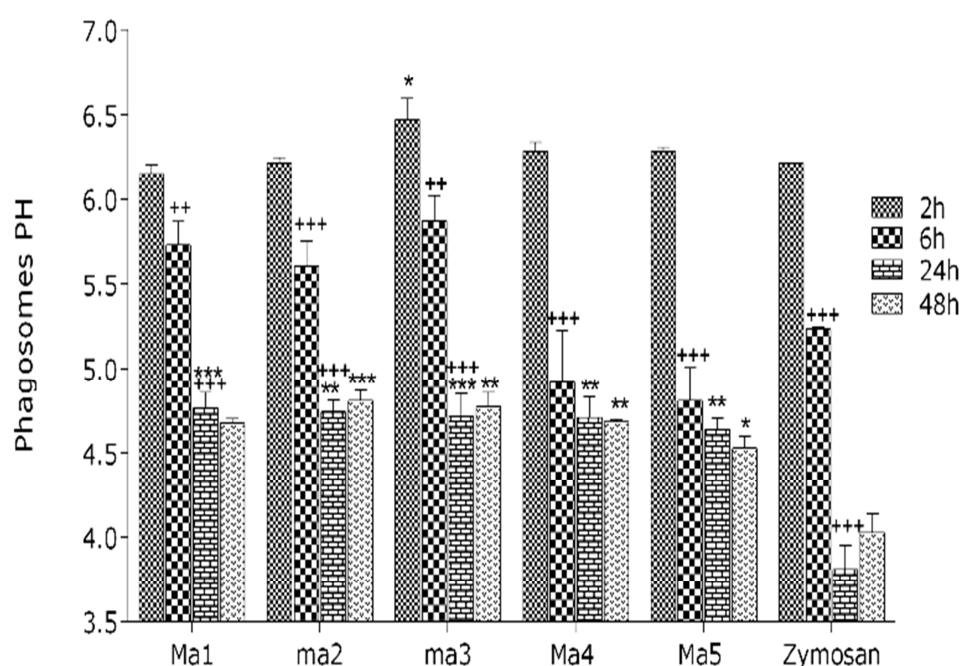


Figure 6.10: pH of HD11 phagosomes containing *M. avium* and Zymosan A. Samples were assayed at 2, 6, 24 and 48h post-infection. Strains *M. avium* Ma1 (NC08562.02), Ma2 (NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08). The data are mean $\pm$ SE, fluorescent intensities determined by FLUOStar from three independent experiments with internal triplicate repeats. Significant differences between time points reported by each mycobacterial strain are indicated by asterisks + = $p < 0.05$, ++ = $p < 0.01$ and +++ = $p < 0.001$. Significant differences between *M. avium* strains and Zymosan A indicated by (*), P -values.

6.2.5 Optimal pH for *M. avium* Ma1-Ma5 growth

To understand the effects of the phagosomal pH on the intracellular survival of five *M. avium* strains, the survival and the growth of the bacterial strains were examined over 48h. The mycobacterial strains were cultured to log-phase in 7H9 broth at defined pH values (4-8). The data found that the bacterial growth was greatly affected by the pH of the media. According to the measured OD at 600nm, the best survival of all five *M. avium* strains in buffered 7H9 broth were

reported at pH 5.5-6 (Figure 6.11) which is very close to the environmental pH encountered in infected HD11 cells at 6h post-infection (figure 6.10).

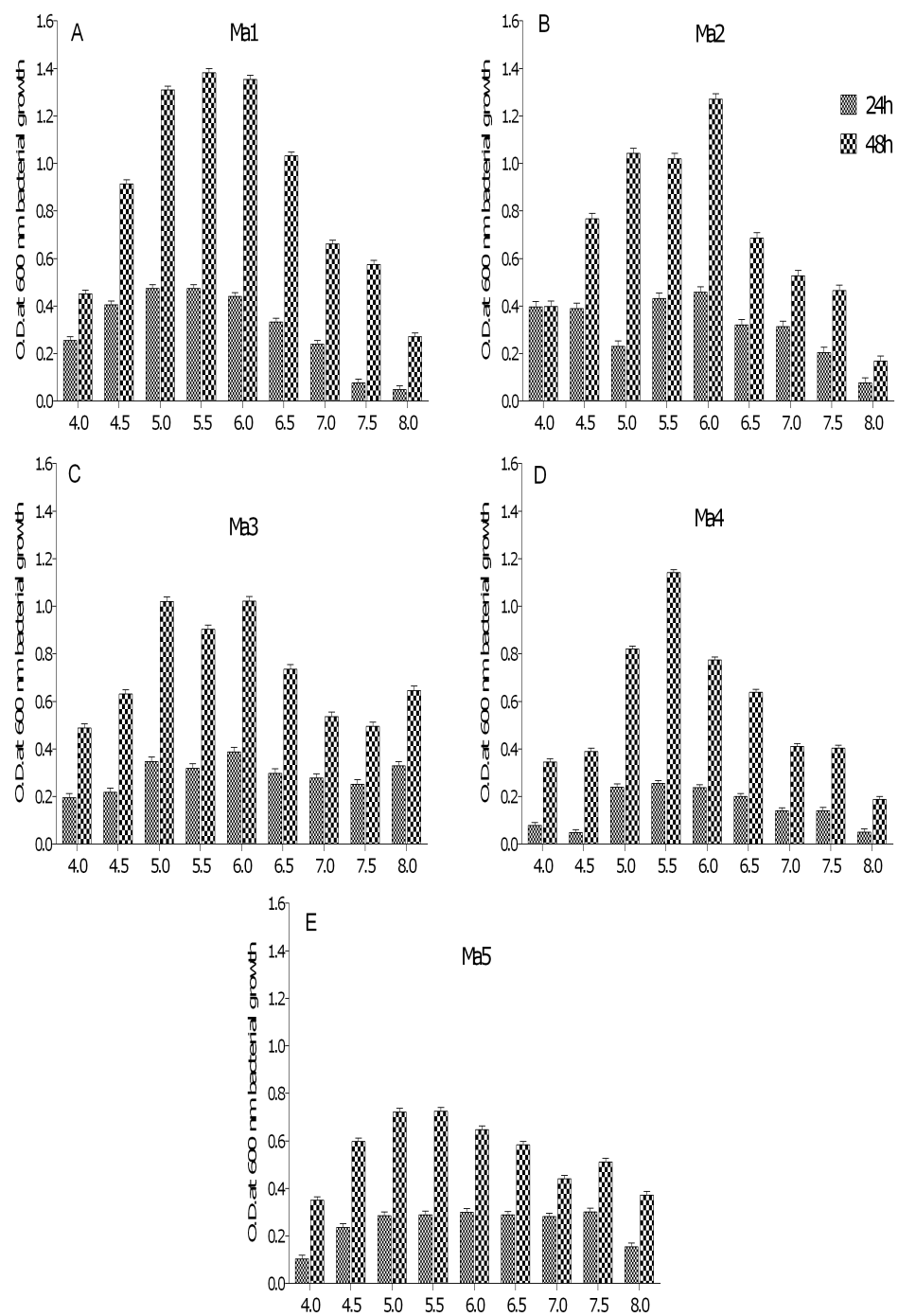


Figure 6.11: Optimal pH for growth of *M. avium*. Strains **(A)** *M. avium* Ma1 (NC08562.02), **(B)** Ma2 (NC08559-07), **(C)** Ma3 (61-3623-05), **(D)** Ma4 (61-4627.08) and **(E)** Ma5 (61-3728.08). Optical densities of the bacterial growths were taken at 600 nm after 24h and 48h of incubation of 500 μ l of 4×10^8 CFU / ml in 7H9 broths adjusted to various pH 4-8 to give an optical density (OD= $\sim$ 0.05 - 0.06 at zero time). Data are shown as mean $\pm$ SE of three independent experiments with two internal replicates per experiments.

6.2.6 Quantitative analysis of intracellular trafficking of *M. avium* by flow cytometry

To study the potential impact on phagosome maturation by the five *M. avium* strains we compared the expression of phagosome maturation proteins during infection compared with a Zymosan A positive control using indirect immunofluorescence techniques. Antibodies were used to monitor expression of phagosomal markers for early endosome (EEA1) and late endosome markers RAB7 and LAMP-1 lysosome markers. Flow cytometry with infected cells showed that at the early time point of 2h post-infection, both bacteria and Zymosan A were associated with the cells expressing the later late endosome marker RAB7 (Figure 6.12). However by 6h and 24h post-infection association with EEA1 was significantly increased in *M. avium* infected cells compared to the Zymosan A suggestive of some modulation effect ($p < 0.05$). The results also showed cells expressing LAMP-1 increased from 2h to 6h but decreased in all but the Zymosan A control group by 24h post-infection. This again suggests some form of modulation of cellular function.

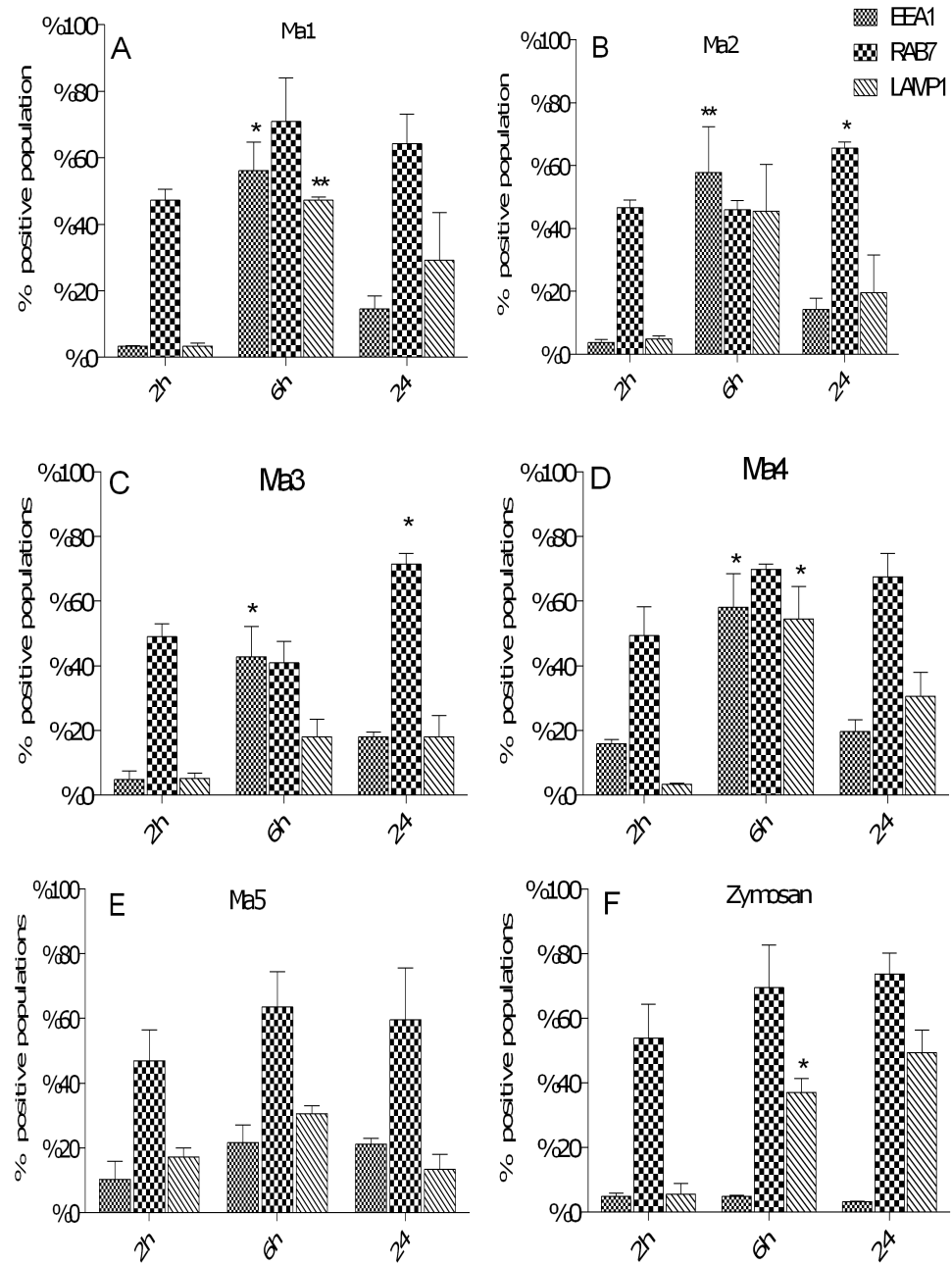


Figure 6.12: Association with phago-lysosomal markers of internalised *M. avium* and Zymosan A in HD11. (A) *M. avium* Ma1 (NC08562.02), (B) Ma2 (NC08559-07), (C) Ma3 (61-3623-05), (D) Ma4 (61-4627.08), (E) Ma5 (61-3728.08) and (F) Zymosan A. Rabbit anti-chicken polyclonal antibodies were used to recognize (EEA1, RAB7 and LAMP1 markers) % of positive populations were determined by flow cytometry. Significant differences reported by each mycobacterial strain between time points of each marker are indicated * $p < 0.05$ and ** $p < 0.01$.

6.3 Discussion

A number of successful intracellular pathogens show the ability to survive and replicate within infected cells by entering via specific receptors that direct bacteria to a compartment which is more conducive to survival (Meena, 2010, Ribet and Cossart, 2015). Here, the role of each receptor in the survival of the bacterial strains was determined in both cell lines over a 48h period post-infection. Competitive receptor blockers were used and compared with non-treated cells as a control.

Blocking of the mannose receptor during infection of THP-1 cells did not have any inhibitory effect in reducing the survival of some of the bacterial strains, namely Ma2 and Ma3, which were increased by 48h post-infection. Similarly the blocking of scavenger receptors did not reduce the numbers of viable Ma3, Ma4 and Ma5 by 48h post-infection. Moreover, CR3 in THP-1 cells did not affect the survival kinetics of some of the bacterial strains, particularly in the case of Ma4, where bacterial invasion was very low. However, the precise role of mannose receptors in bacterial survival is unknown, but it has been suggested that pathogenic mycobacteria utilize mannose receptors to escape from toxic consequences of oxidative burst (Schlesinger, 1993).

In contrast to THP-1 cells, blocking of scavenger-, mannose-, and complement receptors CR3 in HD11 cells showed a clear inhibitory impact on the kinetics of the bacterial survival. The scavenger

receptors were more important than the others. These findings are in line with those already reported which indicated that the use of scavenger receptors was important in subsequent intracellular survival of mycobacteria with *M. bovis* BCG infection of murine cells (Hawkes et al., 2010). Also our data agree with those reported by *M. tuberculosis* in human macrophages, where scavenger receptors were more important than mannose receptors and complement receptors (Zimmerli et al., 1996). Phagocytosis of *M. avium* by human macrophages via mannose receptors enhances the survival of the bacteria, since mannose receptor-dependent invasion interfered with phagosome-lysosome maturation (Shimada et al., 2006). Furthermore, CR3-mediated *M. tuberculosis* ingestion led to decreased nitric oxide and ROS production by mammalian infected macrophages, hindering the maturation process of the phagosomes and inflammatory responses (Sturgill-Koszycki et al., 1997). The present data suggest that the *M. avium* strains use selective receptor-mediated pathways as a survival strategy. In THP1 cells, it is suggested that Ma1-Ma5 can invade the THP-1 via different receptors, but the invasion via specific receptor does not appear to have any impact on the bacterial survival in infected cells.

The cell cytoskeleton is not only important in the invasion mechanisms, but also has been reported to have important functions in the survival of the pathogens (Haglund and Welch, 2011). For instance, disruption of cell microtubules restricts the growth of pathogenic *Salmonella* species within human epithelial

cells and murine macrophages (Guignot et al., 2004). Actin inhibition reduced *Salmonella* growth in infected murine macrophages (Meresse et al., 2001). In this study the influence of the actin and tubulin disruption on *M. avium* survival over 48h in both THP-1 and HD11 cells was investigated. In both cell lines, the data showed that the cytoskeleton played an important role in the intracellular survival of the bacterial strains.

The reduction of bacterial survival in treated cells with cytoskeleton depolarizing agents might be due to the disruption of the bacterial niches which sustain bacterial growth. Previous studies have shown that intact microtubules of murine macrophages are important for phagolysosome maturation (Blocker et al., 1996) suggesting that the increase in the numbers of the viable bacteria in nocodazole treated THP-1 cells is likely due to the effect of the cell tubulin on the maturation processes of phagosomes.

The increase in the numbers of viable bacteria in cytochalasin D-treated HD11 cells is probably, we suggest, as a result of reduction in nitric oxide production. Garcia-Perez et al. (2011) reported that concentrations of nitric oxide produced by murine epithelial cells in response to *M. tuberculosis* infection correlated with the capacity of the bacteria to induce modifications to actin. Murine macrophages treated with cytochalasin D result in reduction in nitric oxide production due to breakdown of iNOS within the cytoskeleton network (Miller et al., 2004). Alternatively, it may be due to

inhibition of iNOS delivery to the bacterial phagosomes. The co-localization of the iNOS with phagosomes containing *M. tuberculosis* is an actin-dependent mechanism. Cytochalasin D inhibits iNOS delivery to the bacterial phagosomes (Miller *et al.*, 2004) or it might be due to inhibition of phagolysosome maturation as a result of actin disruption. Actin disruption causes delay in maturation of phagosomes (Guerin and de Chastellier, 2000).

On the other hand, the nocodazole-treated HD11 cells reduced nitric oxide production, but the bacterial strains were unable to replicate in the treated cells. This is likely due to the influence of the tubulin disruption on accelerating the maturation process of phagolysosomes. It has been reported that the presence of a coat protein (TACO, a tryptophan aspartate-containing coat protein) on the phagosomes containing live *M. bovis* BCG in murine macrophages enhanced the bacterial survival by preventing lysosomal delivery to the phagosomes. TACO is associated with the tubulin networks, disruption of which leads to maturation of phagolysosomes and mycobacterial degradation (Ferrari *et al.*, 1999). Furthermore, disruption of the cell cytoskeleton results in reduction of bacterial invasion in both cell lines. So it is believed that the infected cells with low *M. avium* load can control and limit bacterial growth.

Intracellular localization of *M. avium* and phagolysosomes fusion have been investigated to understand the interactions between the

immune responses of infected HD11 and *M. avium*. Immunofluorescence graphs on the interaction between *M. avium* strains Ma1-Ma5 and the HD11 cytoskeleton demonstrated no bacterial effect on the actin of infected cells compared to the control groups. This finding disagrees with that reported by Guerin and de Chastellier (2000) who found that *M. avium* infection of murine macrophages causes disruption of actin filaments in a similar manner to cytochalasin D treated cells. The difference between the two studies with regards to the bacterial ability in depolymerising actin is probably due to the strains or host cells used, since Guerin and de Chastellier used murine isolate TMC 724 (serovar 2) which is virulent to murine macrophages, whereas here we used chicken and calf isolates in human and avian cells.

The interactions between the bacterial strains and HD11 tubulin showed strain to strain differences; Ma1, Ma2 and Ma3 induced disruption of tubulin in the same pattern as occurred in nocodazole-treated cells. The discrepancies between the bacterial strains are probably related to the survival of the bacterial strains. We also observed that tubulin is co-localized with iNOS and disruption of tubulin interferes with the iNOS functions and thus, Ma1-, Ma2- and Ma3-infected HD11 cells were associated with low nitric oxide production and this may promote the intracellular survival of the bacterial strains. Pathogenic bacteria such as *S. flexneri* (Yoshida et al., 2006), Enteropathogenic *E. coli* (Shaw et al., 2005) and *L. monocytogenes* (Stavru et al., 2011) manipulate the mammalian

host cell microtubule network through effector proteins to survive within the host cell.

Transmission electron microscopy (TEM) data showed that *M. avium* strains were unable to escape from phagosomes to the cytoplasm of infected HD11 cells over 48h post-infection. These findings disagree with those reported by Simeone et al. (2012) when they found that *M. tuberculosis* and *M. marinum* entered the cell cytosol of human macrophages (THP-1) by 24h after initial infection. *M. tuberculosis* relies on ESX-1 secreted protein ESAT-6 to access the cytoplasm of infected cells (Simeone et al., 2012). Co-existence of the *M. avium* within the phagosomes during HD11 infections may be due to the bacteria lacking the ESX-1 secreted protein ESAT-6 (Das et al., 2011). Presence of *M. avium* strains exclusively within the phagosomes suggests that the bacteria hinder the maturation process of phagocytic vacuoles and create a suitable environment to sustain their survival.

The acidification of phagocytic vacuoles containing pathogens is an important antimicrobial mechanism of infected macrophages. Intracellular bacteria exist and replicate within the phagosomes by hindering the maturation of phagosomes through inhibition of the acidification process (Vergne et al., 2003, Meena, 2010). To determine the role of this mechanism in the intracellular survival of *M. avium* strains Ma1-Ma5 in HD11 cells, fluorescent probes (NHS-CF and NHS-Rho) were used to label the bacterial strains and

Zymosan A. This study has used Zymosan A as a control with which to compare the phagosomal pH and intracellular trafficking of *M. avium* in infected HD11 cells. Zymosan A was chosen as a control due to the fact that the Zymosan A can interact with macrophages receptors including scavenger receptors (Jang et al., 2009), mannose receptors (Astarie-Dequeker et al., 1999) and complement receptor CR3 when Zymosan is opsonized with serum proteins (Le Cabec et al., 2000). Furthermore, studies on the phagosomal pH assays of *M. avium*-infected mouse used Zymosan A as control positive (Oh and Straubinger, 1996, Hostetter, 2000).

The phagosomal pH indicates that there were strain to strain differences between strains at different time points post-infection over 48h. The differences between the bacterial strains with regards to the phagosomal pH are probably due to the bacterial interference with the host cell tubulin which hinders phagosomal maturation. Significant differences have also been observed between pH of phagosomes containing Zymosan A and the bacterial strains. These findings are in line with that reported previously (Oh and Straubinger, 1996), who found that live but not heat killed *M. avium* in murine macrophages resided at a pH between 5.5-6.0 after 6h post-infection whereas heat killed *M. avium*, Zymosan A and *Salmonella* Typhimurium resided in a more acidic environment at pH 4.8 suggesting that the live bacteria interfere with the maturation process of the phagolysosomes. Furthermore, it has been shown that live *M. avium* in murine bone marrow macrophages localized

within the phagocytic vacuoles at pH 6.3-6.5, compared to Zymosan A and *Leishmania*, which were found in phagosomes of pH 5.0 (Sturgill-Koszycki et al., 1994). The significant differences between the pH of the phagosomes containing the bacterial strains and Zymosan A suggest this as a survival strategy employed by *M. avium* strains within the infected HD11 cells.

To detect the effect of acidic environment in HD11 cells on survival of *M. avium* strains, the optimal pH for bacterial growth was determined by using 7H9 broths at defined pH values. The data observed that the optimal pH for growth of the bacterial strain is 5.5-6.0. Co-existence of *M. avium* strains within vacuoles at pH 5.5-6.0 and the best growth of the bacterial strains at the same pH suggest that the bacterial strains circumvent low phagosomal pH for their survival.

Using of fluorescence microscopy to analyse samples is time consuming, only a small population of cells can be observed and the weak signals from some antibodies may lead to inaccurate data (Scianimanico et al., 1999). Therefore, flow cytometry was used to determine the intracellular trafficking of phagolysosome. We observed strain to strain differences in the frequency of vacuoles expressing early and late phagosomes markers. In *M. avium* infected cells, the frequencies of vacuoles expressing early phagosome marker EEA1 were markedly increased at 6h after infection with a progressive reduction in acquiring Rab7 (late

phagosome marker) except for strain Ma3. The increases in EEA1 expression by 6h is suggested to be due to the net increases in the bacterial number as a result of bacterial multiplication. These findings are in agreement with those reported for viable *M. tuberculosis* H37Rv and H37Ra in murine macrophages infected by McDonough and his colleagues (McDonough et al., 1993). During the infection, vacuoles with bacterial strains showed the highest rates of Rab7 expressing vacuoles. The rates of LAMP-1 (lysosome marker) expressing vacuoles containing bacterial strains were lower than that of Zymosan A. These findings go in line with those of Oh and Straubinger (1996) who reported only 18% of *M. avium* in murine macrophages are localized within the lysosomes compared to 98% of heat-killed bacteria. Low rates of vacuoles expressing LAMP-1 are probably due to the cell wall structure of the bacteria; it has been seen that *M. tuberculosis* blocks phagosome fusion through its cell wall phosphatidylinositol analogue lipoarabinomannan (LAM) which locks a *trans*-Golgi network-to-phagosome phosphatidylinositol 3-kinase-dependent pathway (Vergne et al., 2003).

The variations between the five *M. avium* strains and Zymosan A in the numbers of vacuoles expressing LAMP-1 suggest that the bacteria interfere with the maturation process of phagolysosomes. It has been seen that pathogenic mycobacteria, *M. tuberculosis* and *M. avium* possess PPE and PE gene families, a gene member of which has been found to be responsible for preventing of phagosome-

lysosome fusion in *M. avium*-infected human macrophages (Li et al., 2005). The increased rates of Rab7-expressing vacuoles during infection supports the data on the optimal pH for the bacterial growth, as it has been seen that the early vacuole pH is measured at values between 6.1 and 6.5 and late endosomes at 5.5 (Xu et al., 1994, Sturgill-Koszycki et al., 1994, Sorkin and von Zastrow, 2002).

Taken together, the data presented above suggest that the *M. avium* can use the same intracellular survival mechanisms in chicken cells as other pathogenic mycobacteria, and especially similarly to *M. tuberculosis* in mammalian cells. *M. avium* strains Ma1-5 can use more than one strategy simultaneously to survive in the HD11 cells.

7.0 Discussion

The main aims of this study were to determine the mechanisms *M. avium* subspecies *avium* uses to invade and survive within the avian macrophage cells compared to human macrophage. This data could then be compared to the published data on equivalent processes in *M. tuberculosis* and *M. bovis* infection of mammalian cells.

7.1 Invasion and survival

Macrophages are the most critical host cells for mycobacteria, where the bacteria are, to some extent, able to evade the host adaptive immune system and undergo intracellular replication. To enter the host cells, mycobacteria must bind to the surface of the cells (Alberts et al., 2002, Stokes et al., 2004). Bacteria can bind to serum proteins C3bi and enter to phagocytic cells via complement receptor CR3 (Bermudez et al., 1991, Ferguson et al., 2004, Ratledge and Dale, 2009) or interact directly with carbohydrate recognition domains through mannose-capped lipoarabinomannan and phosphatidylinositol mannosides (Kang and Schlesinger, 1998, Kang et al., 2005). Bacterial cell surface sulfolipids can also bind directly to the macrophage scavenger receptors and promote invasion (Ernst, 1998, Schäfer et al., 2009). The subsequent receptor mediate entry of the bacteria into macrophages can then be either an actin or tubulin-dependent phagocytic process (Haglund

and Welch, 2011). The exact mechanisms by which invasion occurs could have a profound impact on the final intracellular compartmentalisation of the bacteria and therefore on their survival.

The invasion and survival of *M. avium* strains were investigated in human macrophage-like cell lines, THP-1 (human) and HD11 (chicken). The cells were infected with five different strains of *M. avium*, two poultry isolates (Ma1 and Ma2) and three bovine isolates (Ma3, Ma4 and Ma5). Here we have shown that all of the bacterial strains were able to invade and replicate within the host cells. There were strain to strain differences in the invasion and survival levels of the host cells with the highest invasion rates in THP-1 and HD11 cells reported for Ma5. Ma1 and Ma3 which were the most resistant strains to the intracellular hostile environment of the infected macrophages. The kinetics of the intracellular survival of the bacterial strains were different between human (THP-1) and the avium (HD11) cells over the 48h infection period. While we only assessed a limited number of strains the source of the *M. avium* strain did not appear to correlate with the invasion or intracellular survival.

Invasion of macrophages was differentially enhanced in the presence of the different host sera on the different host cells. Invasion of THP-1 was increased in the presence of human serum compared to the calf and chicken sera which were restricted for

human cells. Conversely human sera much reduced the invasion of the bacterial strain in avian cells compared to calf and chicken sera.

Then role of serum in invasion is not unexpected and is in accordance with the results reported for *M. avium* invasion of human cells in the presence of and absence of human opsonic components of the serum (complement, fibronectin, C-reactive protein and fibrinogen) (Bermudez et al., 1991). *M. avium* strains were able to activate the complement system through both classical and alternative pathways. The results of this study are also in agreement with data on complement and invasion of *M. tuberculosis*, *M. leprae*, and *M. avium* via the alternative pathway even in the absence of C4 (Schorey et al., 1997).

The discrepancies between the results for different sera used in bacterial opsonisation and invasion are most probably due to the host species-specific differences in the structure of the complement components. C3 in chicken serum is found to be similar in function and structure to that found in the mammalian sera, it has been claimed and is thought to exist in three different molecular forms compared to human C3 (Laursen and Koch, 1989).

Human sera had a significant inhibitory effect on *M. avium* invasion of HD11 cells in the presence of chicken- and foetal calf serum. As it can promote invasion in human cells it would suggest it binds the *M. avium* but then cannot interact with the relevant complement receptors thus becoming a competitive inhibitor for invasion. In

contrast, chicken sera had no significant inhibitory effect on uptake *M. avium* by THP-1 cells in the presence of human serum. Either these serum components can interact with THP-1 cells more readily or they are out competed for binding for the bacteria.

Other potential differences between the roles of the sera could contribute to the effects seen is the physiological levels of IFN- γ in human sera. It has been shown that murine IFN- γ down-regulates mannose receptor expression in murine macrophages (Harris et al., 1992, Stahl and Ezekowitz, 1998, Stein et al., 1992). It has also been reported that human IFN- γ interfered with normal function of complement receptors in human monocyte-derived macrophages leading to decreased uptake of *Mycobacterium leprae* (Schlesinger and Horwitz, 1991). Thus the potential exists for the inhibition due to human IFN- γ effects on HD11 cells. However human and chicken interferons are not directly interchangeable in their stimulatory function so human IFN may play not a part in the reduction in invasion.

The freezing of both human sera and chicken sera reduced their opsonic activities compared with fresh sera. In contrast, the effect observed on the opsonic activities between fresh and frozen foetal calf sera on the bacterial invasion rates by the host cells was not significant. The variations observed between fresh and frozen sera are probably be due to denaturation of complement component C4, resulting from the freeze-thawing process (von Zabern and Gigli,

1982) or loss of MASP-1 and MASP-2 activity through freezing which are required for activation and cleavage C4 and C2 (Presanis et al., 2004).

The roles of individual mannose-, scavenger- and complement CR3 receptors in *M. avium* invasion into the host cells were investigated by using competitive chemical ligands. In THP-1, the data showed that mannose receptors were the dominant receptor while in HD11 cells scavenger receptors (SRs) dominated the entry of the bacterial strains except Ma4. The THP-1 data agree with studies on invasion of *M. tuberculosis* into human macrophages which was strongly mannose receptor-dependent (in the absence of human sera) and complement receptor (in the presence of human sera) (Schlesinger, 1993, Zimmerli et al., 1996) while the invasion of in *M. bovis* was greatly reduced in scavenger receptor deficient mice (Hawkes et al., 2010). Simultaneously blocking mannose-, scavenger- and complement CR3 receptors did not result in complete inhibition of bacterial invasion in both cell lines indicated that *M. avium* may also invade through other undefined receptors. This is in line with invasion data for *M. bovis/tuberculosis* which will also use the alternate scavenger receptors when the bacterial complement and mannose receptors are blocked (Zimmerli et al., 1996).

Mycobacteria invade human macrophages via mannose and complement CR3 receptors to avoid induction of reactive oxygen species and survive within the infected macrophages (Astarie-

Dequeker et al., 1999, Tessema et al., 2001a). It has been reported that mannose-mediated *M. avium* invasion in human macrophages interferes with phagosome-lysosome maturation and enhances the intracellular survival of the bacteria (Shimada et al., 2006, Schlesinger, 1993, Welin et al., 2008, Rajaram et al., 2014). Furthermore, it has been reported that scavenger receptors enhance the intracellular survival of *M. bovis* BCG in murine cells; the bacteria use the receptor to evade from the immune responses of the host through interfering with nitric oxide induction (Hawkes et al., 2010).

Receptor mediated entry will require remodelling of the cell cytoskeleton. It is well known that the phagocytosis of mycobacteria into the host is an actin-dependent process (Ferrer et al., 2009) however, the role of tubulin in the early phagocytosis is unknown. The data presented show that both actin and tubulin are crucial for internalization of *M. avium* of both human and avian cell lines. Actin appears to be more dominant in the invasion process than tubulin. There were strain to strain differences and cell to cell variations. For instance, a combinatorial effect of cytochalasin D or nocodazole on CR3-mediated invasion of Ma5 into THP-1 cells and nocodazole on CR3-mediated phagocytosis of Ma2 by THP-1 was not additive, but the invasion was lower than that observed by blocking of cytoskeleton alone. The effect of simultaneous blocking of mannose receptors and tubulin disruption on *M. avium* invasion into HD11 cell

was not synergistic, implying that mannose receptors mediated the bacterial invasion is not a tubulin-dependent mechanism.

The decreases in the invasion rates of the bacterial strains due to actin and tubulin disruption are probably due to the dysfunction or interference with the optimal function of the cell receptors. It has been reported that the intact actin is required for optimal mannose-, scavenger- and complement CR3 receptors dependent internalization of particles by human macrophages (Al-Jarallah et al., 2014, Newman et al., 1991, Allen and Aderem, 1996, Defife et al., 1999). It also has been reported that complement and scavenger receptors of human macrophages need intact tubulin for phagocytosis (Sulahian *et al.*, 2008) whereas it has been shown that the invasion of *M. tuberculosis* of human fibroblasts does not require tubulin reorganization although these cells are not professionally phagocytic (Garcia-Perez et al., 2011)

To our knowledge, the functional interactions between the cell cytoskeleton and the cell surface receptors and their effects on the bacterial invasion have not been investigated before. It is thought that the inhibitory effects of the combined treatments would have synergistic effects on the bacterial invasion. However not all blocking was synergistic and we observed differences between human and chicken cells and the strains assessed.

The non-synergistic effects of a simultaneous blocking of receptor and cell tubulin or actin on Ma5 of THP-1, but not HD11 cells, were

probably the result of bacterial ability to interact with different receptors on the phagocytic cells. Ma5 used scavenger-, mannose- and complement CR3 receptors nearly at the same levels to invade the host cells. Cell to cell variations with regards to the effects of simultaneous blocking of an individual receptor with actin or tubulin on the bacterial invasion might be due to the structural differences between human and avian cells. It has been reported that the mammalian multigene families encoding actin cytoskeleton are more complex and larger than those in chicken cells (Chang *et al.*, 1984) suggesting that requirement for cytoskeleton remodelling may differ between avian and mammalian cells.

7.2 Survival mechanisms

7.2.1 Innate antimicrobial mechanisms

Macrophages play a critical role in the innate immune response against mycobacterial infection. Data on the role of nitric oxide (NO) and reactive oxygen species (ROS) during *M. avium* subspecies *avium* of chicken macrophages is limited. Therefore, the present study aimed to investigate the role of nitric oxide and reactive oxygen species in intracellular survival of *M. avium* using both HD11 cells and THP-1 cells.

Macrophage cells can be activated by addition of LPS, INF- γ and Tumour Necrotizing Factor and by infecting bacteria (Lewis *et al.*, 1990). Activated macrophages kill intracellular pathogens via

production of reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) (Chan et al., 1992, Denis, 1991, Bartos et al., 2004).

Infection of both HD11 and THP-1 cells with *M. avium* induced the production ROS while only HD11 produced nitric oxide. THP-1 cells are known to have limited production of nitric oxide (Pérez-Pérez et al., 1995, Rajora et al., 1996). There were strain to strain differences in the number of bacterial survival and in nitric oxide production at 24h and 48h after infection. The reduction in intracellular bacteria cells correlated with increased levels of nitric oxide production in the infected HD11. This suggested that reduction in bacterial survival was due to the level of nitric oxide production. The role of nitric oxide in intracellular survival of the *M. avium* strains in HD11 cells was further investigated by using the nitric oxide inhibitor *L*-NMMA and inducer rchIFN- γ and also by detecting the iNOS-co-localization rates with bacterial strains over 48h post-infection. We observed that the intracellular survival of *M. avium* strains were greatly reduced by increased nitric oxide production. These results are in agreement with that reported by Flesch and Kaufmann (1987) who found an inhibitory effect of nitric oxide induced by IFN- γ on the intracellular growth of *M. bovis* BCG and *M. tuberculosis* H37Rv in bone marrow-derived murine macrophages.

To understand to what extend phagosomes containing *M. avium* co-localized with iNOS and its relationship with nitric oxide production

by infected HD11, host species-associated representative strains *M. avium* Ma1 (avian isolate) and Ma4 (calf isolate) were chosen and the immunofluorescence technique was used, *M. avium*-iNOS co-localization rates were investigated over 48h. The data showed that the delivery of iNOS to the phagosomes containing Ma4 was faster than that of Ma1 and the percentage of the co-localized Ma1 was lower than that of Ma4, particularly during the first 24h post-infection. Nitric oxide production by Ma4-infected HD11 cells was also significantly higher than that of Ma1 infected HD11 cells. These findings suggest that Ma1 may inhibit the delivery of iNOS to its phagosomes and or disrupt its induction. It was observed that Ma1, Ma2 and Ma3 caused disruption and aggregation of cell tubulin compared to strains Ma4 and Ma5 which were unable to induce depolymerization. Additionally, disruption of tubulin with nocodazole significantly reduced the ability of HD11 cells to produce nitric oxide production in response to LPS. We propose that this disruption by strains Ma1-Ma3 plays a key role in the bacterial survival by inhibiting and disrupting nitric oxide production. It is interesting to speculate that the host source or specific group of *M. avium* have distinct properties which mean they behave differently and have different virulence on hosts. The avian isolates (Ma1 and Ma2) both showed reduced nitric oxide responses as did our calf isolates. While a serological differentiation had been developed in (Reznikov and Dawson, 1973, Klausen et al., 1997) we were unable to source relevant reagents to assess the strains. Therefore, in any future

studies we would focus on developing a robust typing based on sources and virulence of a range of strains and their induction of nitric oxide.

The reductions in the numbers of viable bacteria were not only observed in infected HD11 cells, but also in THP-1 cells where no nitric oxide was produced, but the bacterial numbers were decreased at 48h post-infection. It is widely accepted that ROS plays a significant role in restriction of intracellular survival of mycobacteria (Edwards et al., 2001). Therefore, the present study aimed to study the role of ROS in *M. avium* survival in both HD11 and THP-1 cells. Our results contrasted strongly with those which may be expected with *Enterobacteriaceae* where the usual role of ROS is to reduce survival of intracellular bacteria. Our data indicated that *M. avium* grew well in an oxidizing (ROS expressing) environment compared with antioxidant groups (reduced ROS) in both cell lines. These data are however in agreement with those reported for by others intracellular survival of *M. abscessus* in THP-1 cells which also indicated that ROS expression correlates with increased survival of the internalised bacteria (Oberley-Deegan et al., 2010a). In contrast, it has been reported that the growth of *M. bovis* BCG is inhibited in the presence of glutathione (Green et al., 2000, Venketaraman et al., 2003a). It has also been reported that the activated macrophages produce high concentrations of antioxidants during generation of ROS and RNS (Seres et al., 2000, Venketaraman et al., 2003b).

7.2.2 Survival strategies utilized by *M. avium*.

M. tuberculosis can successfully manipulate the hostile environment of mammalian macrophages and maintain bacterial viability via different strategies. In the context of both *M. avium* and the chicken macrophages it was unknown whether the bacteria use the same strategies as other pathogenic mycobacteria to subvert and or avoid killing.

The invasion of the host via a specific receptor could have significant impact on the fate of the ingested bacteria as they lead to different internal compartments with different environment and as described above some strains can disrupt the cytoskeleton which is vital for maturation of the phagosome.

The roles of the specific mannose-, scavenger- and complement CR3 receptors in bacterial survival were investigated in both cell lines over a 48h period. Previous studies on *M. tuberculosis* have shown that invasion via mannose receptors was linked to enhanced survival of bacteria through an inhibition of phagosome-lysosome maturation (Shimada et al., 2006). CR3 dependent bacterial invasion has been linked to decrease nitric oxide and ROS production and also by hindering the maturation process of the phagosomes and inflammatory responses (Sturgill-Koszycki et al., 1997).

In this study we found that the bacterial invasion of THP-1 cells via mannose receptors did not have any impact on the survival of some of the bacterial strains, namely Ma2 and Ma3, which showed

increased bacterial viable numbers by 48h post-infection. When we blocked scavenger and complement CR3 receptors this also did not show any impact on the bacterial survival over 48h when compared to untreated THP-1 cells.

In contrast to THP-1 cells, the intracellular survival of *M. avium* strains within the infected HD11 cells did change when specific receptors were blocked. Blocking of individual scavenger-, mannose- and complement CR3 receptor enhanced HD11 cells to reduce *M. avium* invasion and control the bacterial growth. These findings are in agreement with what has been reported by others (Hawkes et al., 2010), where *M. bovis* BCG survival of in murine cells found to rely on the bacterial invasion via scavenger receptors.

The correct function of the host cell cytoskeleton can also affect survival of the pathogen. We have shown that the cytochalasin D - treated HD11 cells significantly reduced nitric oxide production in response to LPS compared to the non-treated LPS challenged cells. In this study, the data showed that intact actin and tubulin played an important role in the survival kinetics of the bacterial strains; inhibition of actin in THP-1 cells lead to a reduction in bacterial survival while inhibition of actin modelling in HD11 lead to an increase in survival of bacteria. We suggest the reduction in nitric oxide by cytochalasin D treated HD11 is a major contributing factor to the bacterial survival in HD11 cells. A potential reason decreases within the numbers of the viable bacteria in infected THP-1 treated

with cytochalasin D might be due to the disruption of the bacterial niches which sustain bacterial growth.

The increase in the intracellular survival of the bacterial strains in nocodazole treated THP-1 cells is probably due to impact of the cell tubulin on the maturation process of phagosome-lysosomes.

The nocodazole-treated HD11 cells reduced nitric oxide production, but the survival of the bacterial strains were reduced in the treated cells. This may be due to the disruption of the niches that maintained bacterial survival, or it may have been as a result of the effect of tubulin disruption on accelerating the maturation process of phagolysosomes since it has been reported that the presence of TACO inhibits lysosomal delivery to the phagosomes. TACO is associated with the tubulin networks, disruption of this association which leads to maturation of phagolysosomes and mycobacterial degradation (Ferrari et al., 1999). In addition, we have found that the bacterial invasion of THP-1 and HD11 cells treated with actin and tubulin depolarizing agents were greatly decreased. So we suggest that the infected cells with a low bacterial load can control and restrict bacterial survival.

To determine if the bacteria could modify the maturation of their intracellular niche we looked at both phagosome acidification and developmental markers of the phagosome. Inhibition of phagosome acidification is a critical intracellular survival strategy utilized by *M. avium* in HD11 cells. We observed a difference in reduction of

phagosome pH for all bacteria assessed compared to the positive phagocytic control of Zymosan A. We also observed between the strains at different time points post-infection. Ma1, Ma2 and Ma3 were found to have a much higher final pH (5.5-6.0) by 6h compared to Ma4 and Ma5 (pH<5). The differences between the bacterial strains may be related to an ability to disrupt tubulin as intact microtubules are required for phagosome-lysosome fusions (Blocker et al., 1996). These findings are in line with those reported for infections of murine macrophages by *M. avium* where live bacteria maintained a higher pH compared to Zymosan A and killed bacteria (Oh and Straubinger, 1996). Our data suggest a similar survival strategies are employed by *M. avium* within the infected HD11 cells. The role of this pH modulation in survival was further supported by our culture data on the optimal pH for *M. avium* growth. The bacterial strains showed optimal growth at pH 5.5-6, which is nearly the same pH which *M. avium* strains encounter within phagocytic vacuoles by 6h post-infection. We therefore believe that that the bacterial strains modify the phagosomal maturation and hence pH for their survival.

To test this we looked at the impact of infection on the maturation state of the phagosomes using flow cytometry. We observed strain to strain differences in the frequency of vacuoles expressing early and late phagosomes markers. Ma5 was found with the highest rates of early EEA1 expressed vacuoles which refers to the bacterial invasion rate and this supports our preliminary data on the invasion

rates of the bacterial strain in infected HD11 cells. The increased rates of newly formed vacuoles expressing early phagosomes marker, EEA1, at 6h post-infection also support the intracellular survival hypothesis that *M. avium* replicated by 6h of infection in HD11 cells. The net increases in the number of vacuoles expressing EEA1 were due to the increases in the number of the viable bacterial strains Ma1-Ma5. The bacterial strains Ma1-Ma5 were able to maintain the frequency rates of RAB7-expressing vacuoles at high levels compared to that for Zymosan A during infections. The variation between the 5 *M. avium* strains and Zymosan A suggests that the bacteria interfere with the maturation process of phagolysosomes. Survival of *M. avium* strains within high rates Rab7-expressing vacuoles support the phagosomal pH data where the bacterial strains kept their phagosomal pH above 5 compared to that of Zymosan A pH>4. The early vacuole pH measured at values between 6.1 and 6.5 and late endosomes at 5.5 (Xu et al., 1994, Sturgill-Koszycki et al., 1994, Sorkin and von Zastrow, 2002).

7.3 Summary and Conclusions

Taken together, we observed strain to strain variation in initial invasion and survival suggestive of different interactions between different *M. avium* strains. These strain to strain differences were independent of the source of the isolate. However the typing of these strains to specific host niches is unclear and hard to determine. We also observed subtle differences dependent on the

host species of macrophages studied when infected with the same bacteria suggesting some differences between species for dominance of invasion processes. Serum factors were clearly host specific, with both calf and poultry serum providing an increase in invasion of avian cells not seen with human cells and with human serum providing an increase in invasion of human cells not seen with avian cells. There were clearly multiple different receptor-mediated routes that the mycobacteria could employ to enter the cells and entry via different receptors could have significant effect on the bacterial survival within the HD11 cells. We propose that *M. avium* strains utilise specific receptors to enter the HD11 cells in order to maximize survival. In contrast, in THP1 cells, it seems that receptor selection has a more limited impact on subsequent events. The cell cytoskeleton is not only important for bacterial invasion, but also plays a role in determining the intracellular fate of the bacteria. It appears that some strains disrupt host cell cytoskeleton/tubulin to their survival advantage. Post-invasion nitric oxide and ROS play different roles in the intracellular bacterial survival. Increased nitric oxide correlated with killing of bacteria while ROS enhanced *M. avium* survival.

Taken together the work presented here suggest while there is some variation in receptor preference between hosts, *M. avium* uses the same general strategies to survive within the infected cells as *M. tuberculosis* does in mammalian cells. That these strategies relate to differences in invasion through different receptors and that

modification of phagosome maturation is an important feature of intracellular survival of the bacteria

7.4 Future work

Future studies will aim to develop a robust typing based on sources and virulence of a range of strains as we were unable to source relevant reagents to assess the strains based on serological differentiation. It also would be valuable to investigate the reasons behind the inhibitory effect of human sera on the bacterial invasion into HD11 cells and to investigate why serum factors were host specific. Another interaction study between *M. avium* and avian cells should include additional time points to show the fate of the bacterial strains beyond 48h since the survival data found variation between the bacterial strains. In addition, it would be necessary to determine what factors are involved in the exclusion of iNOS from the bacterial phagosomes in infected HD11. It is generally accepted that antibody-mediated immune response plays a pivotal role in controlling bacterial infection, whereas, the role of antibody mediated immune response in mycobacterial infection is still elusive. However, evidence for the protective role of antibodies against mycobacterial infection in mammalian cells has been reported (López et al., 2010, Achkar et al., 2015), but in the context of both *M. avium* and the chicken macrophages it is unknown whether the antibodies and FcR engagement will subvert the strategies by which *M. avium* strains evade lysosomal degradation. Therefore, it is important to understand the effect of antibodies and FcR

engagement in intracellular survival of *M. avium* within the avian cells.

8.0 Appendices

8.1 Calibration between the bacterial counts and the optical density of different strains of *M. avium*

For infection experiments and to determine the exponential growth phase of each strain, growth patterns for different serotypes were determined using the serial dilution/viable colony count and spectrophotometric methods (OD) (Figure 8.1). A calibration graph was designed between the $\log_{10}$ counts and the optical density and the curve was used to estimate the infection dose (1:10) for each individual strain.

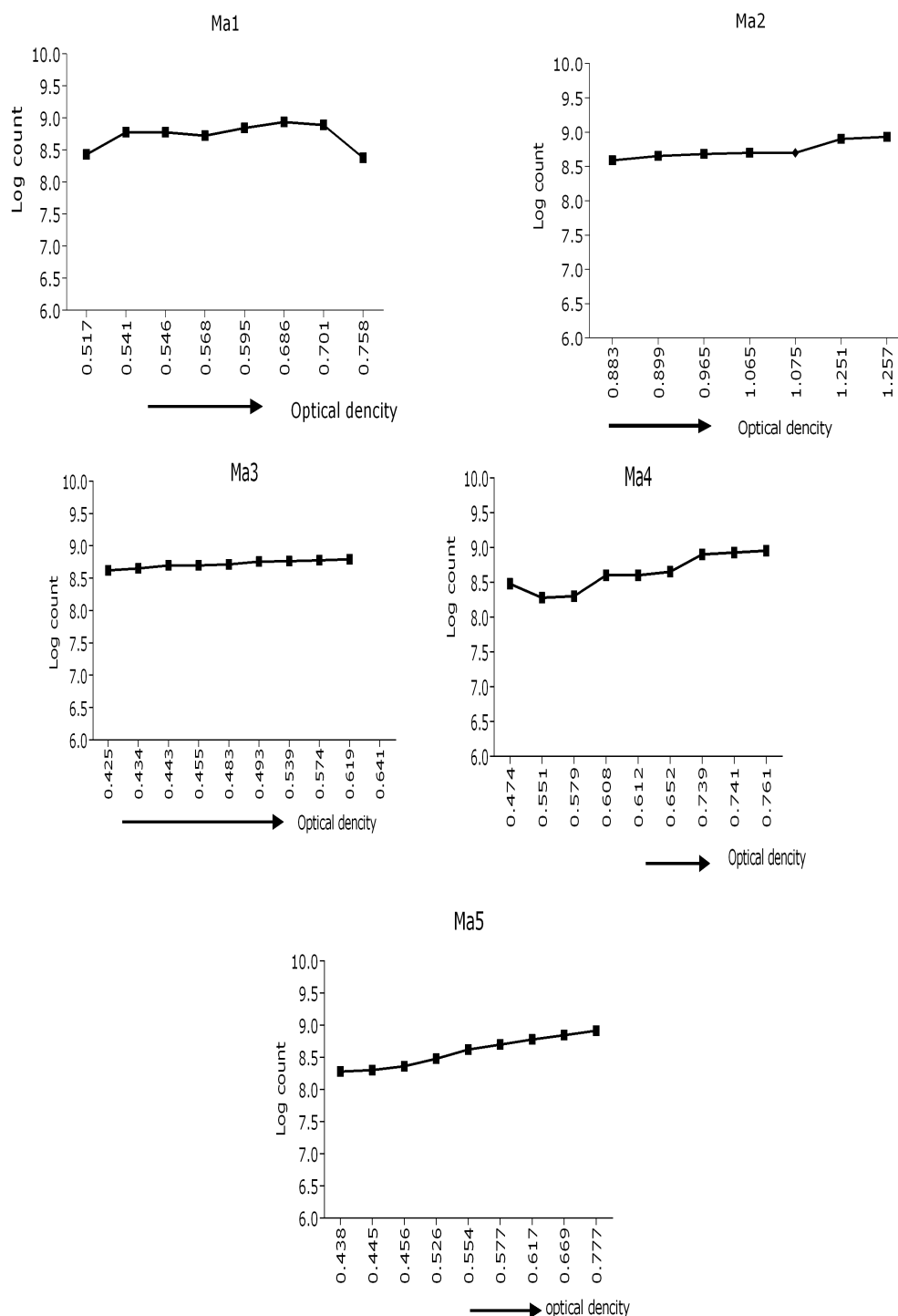


Figure 8.1: Calibration graphs between the bacterial counts and the optical density of different strains of *M. avium* which were used for estimation of the infection doses. Strains Ma1 (*M. avium* NCo8562.02), Ma2 (*M. avium* NC08559-07), Ma3 (61-3623-05), Ma4 (61-4627.08) and Ma5 (61-3728.08).

8.2 Sodium nitrite standard curve

For each assay a standard reference curve of nitrite was prepared for accurate measurement of nitrite concentration. For that seven points of a double fold serial dilution were used in triplicate. The standard was prepared from 1ml of a 100 μ M of nitrite solution by diluting the provided 0.1M sodium nitrite standard 1:1,000 in RPMI. A standard curve plotted in Excel using the trend line function and setting y-intercept to detect the actual value of the produced levels of nitrite.

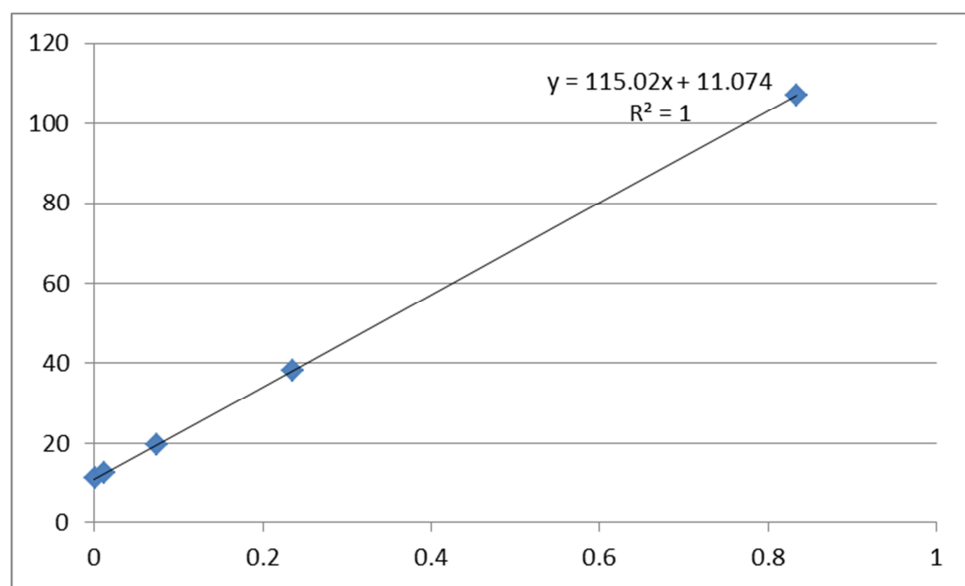


Figure 8.2: Representative sodium nitrite standard curve.
An example of sodium nitrite standard curve for determination of nitrite concentration in HD11 cells supernatants.

8.3 Enumeration of intracellular *M. avium* Ma1-Ma5

To determine the number of bacteria in individual HD11 cell at different time points over 48h post-infection, the infected cells on the cover slides were washed, fixed, permeabilized then stained with

an Auramine O-rhodamine for 15 min. The intracellular *M. avium* Ma1-5 were revealed bright green coloured bacilli within blue ground of DAPI stained cells (Figure 8.3). The intracellular bacteria were enumerated using a LEICA DM 5000B Fluorescent microscope with a 63 X 1.25 lens

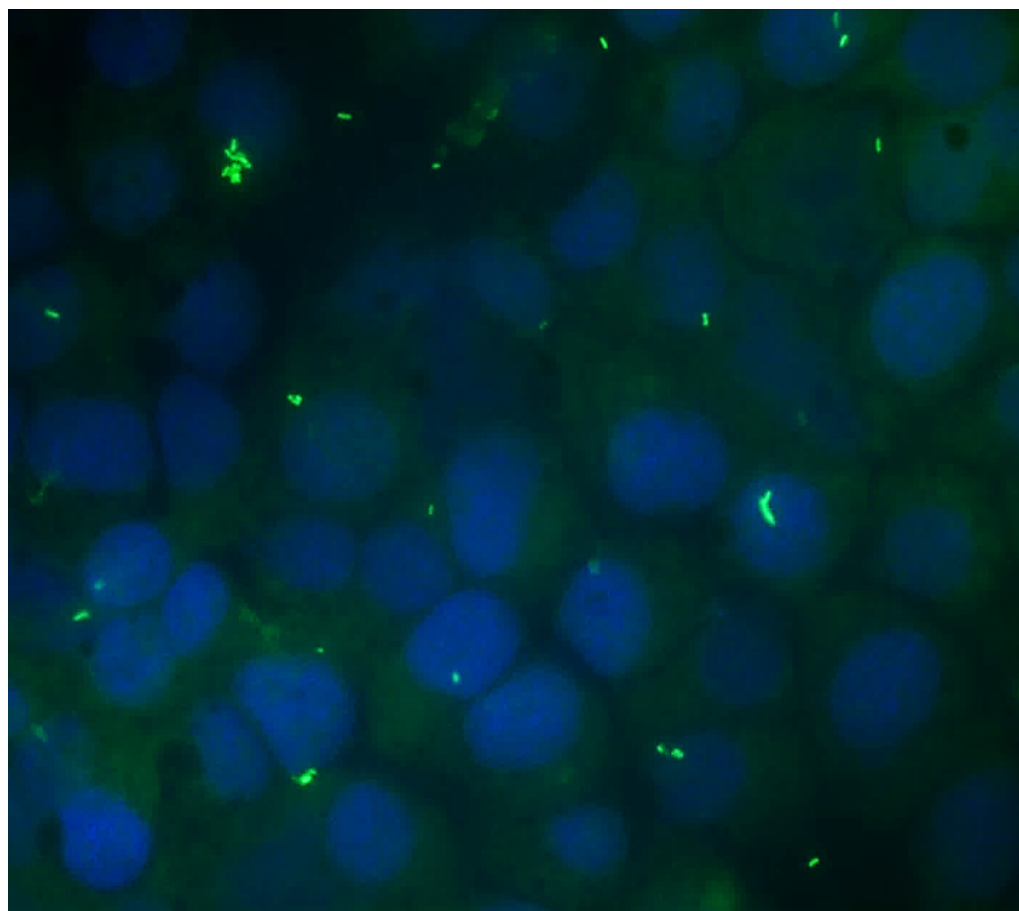


Figure 8.3: Represented image of *M. avium* in infected HD11 cells (2 hours). These images were used to calculate the number of cells containing single, double and more than two bacteria. 50 fields in cover slips for each strain were counted in triplicate wells of three independent experiment of view were assessed in generation of bacteria data. *M. avium* were stained by Auramine O-rhodamine (AO) staining kits and visualized using LEICA DM 5000B Fluorescent microscopes with a 63 X 1.25 lens.

8. 4 Optimization of antioxidants

In this study, the concentrations of the antioxidants to inhibit ROS production by THP-1 and HD11 were chosen according to the minimum cytotoxicity effects with a minimum ROS production. Different concentration of NAC and DPI were tested either alone or together to find out the optimized concentration of antioxidants before assessing the ROS effects on the intracellular survival of *M. avium* in THP-1 and HD11 cells (Figure.8.4, 8.5, 8.6 and 8.7)

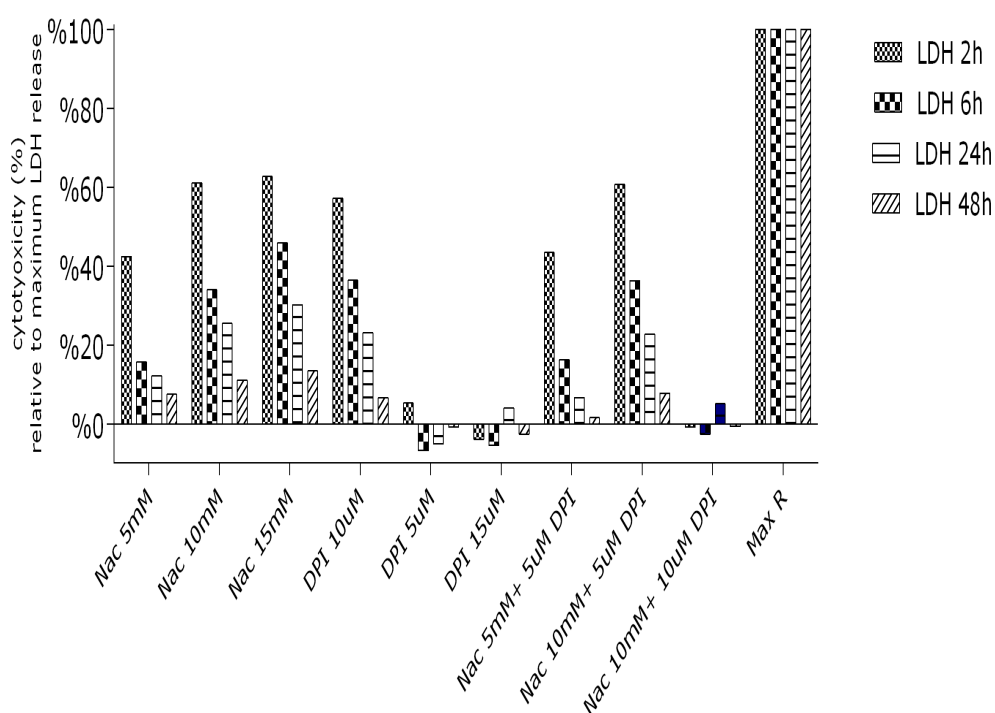


Figure 8.4: Cytotoxicity of *N*-acetyl-*L*-cysteine and diphenyleneiodonium chloride on THP-1 cells. The cells were treated with different concentrations of antioxidants and LDH was determined at 2hrs, 6hrs, 24hrs and 48hrs post-treatment.

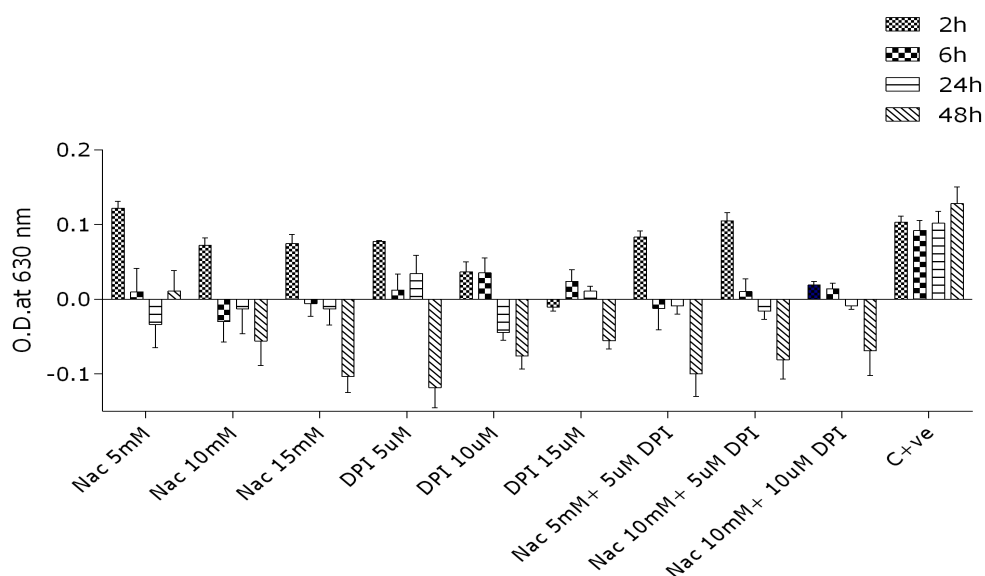


Figure 8.5: Optimization of antioxidants *N*-acetyl-*L*-cysteine and diphenyleneiodonium chloride on reducing ROS production by LPS treated THP-1 cells at different time point post treatment. ROS level is expressed as increase of the optical density at 630 nm (OD690 nm) above negative control. Assays were done at 2, 6, 24 and 48h post-treatment.

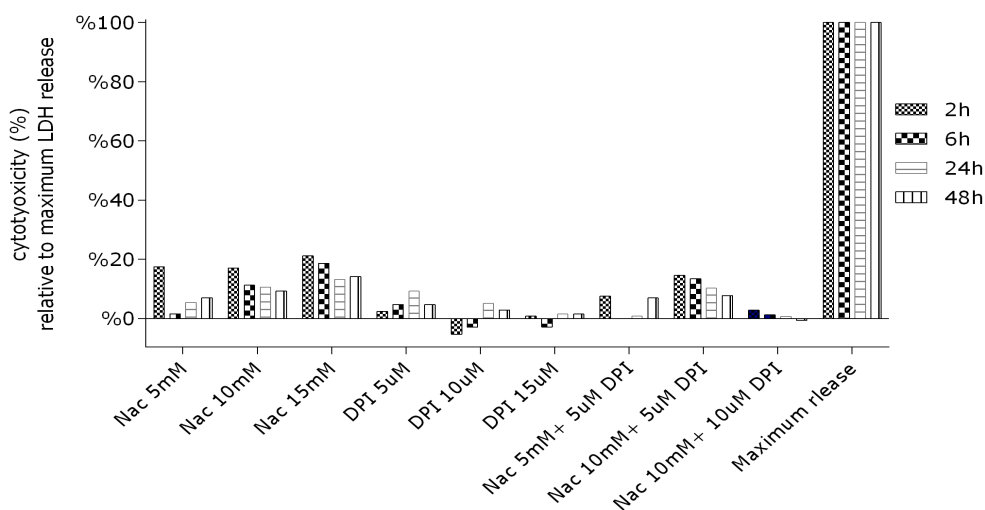


Figure 8.6: Cytotoxicity of *N*-acetyl-*L*-cysteine and diphenyleneiodonium chloride on HD11 cells. The cells were treated with different concentrations of antioxidants and LDH assays were done at 2, 6, 24 and 48h post-treatment.

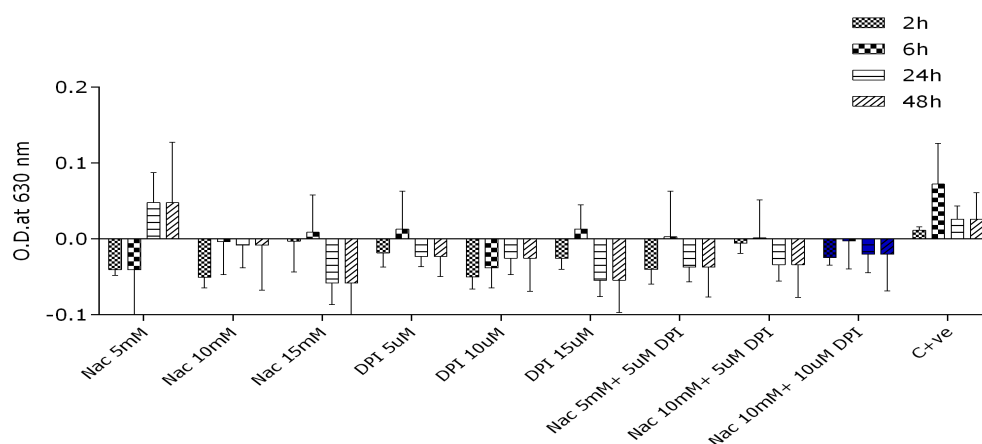


Figure 8.7: Optimization of antioxidants *N*-acetyl-*L*-cysteine and diphenyleneiodonium chloride on reducing ROS production by LPS treated HD11 cells. ROS level is expressed as increase of the optical density at 630 nm (OD₆₃₀ nm) above negative control. Over 48h post infection. Assays were done at 2, 6, 24 and 48h post-treatment.

8.5 Optimization of rchIFN- γ for induction of nitric oxide

The concentration of the nitric oxide stimulator recombinant chicken gamma interferon (rchIFN- γ) to stimulate HD11 was chosen according to its cytotoxic effects to HD11 cells (Figure 8.8 and 8.9). Nitrite production levels compared with control of non-treated cells were challenged with 100 μ l of LPS at 50 μ g/ml and negative controls wells of mock-infected (PBS). For nitric oxide production, HD11 cells were incubated with different concentrations of recombinant chicken IFN- γ for different times points over 24h (Figure 8.8). Following the incubation period at different time points, the supernatants were kept for nitric oxide and cytotoxicity assays. The best results were reported when HD11 cells were incubated with 100 IU of rchIFN- γ for 2h and challenged with LPS.

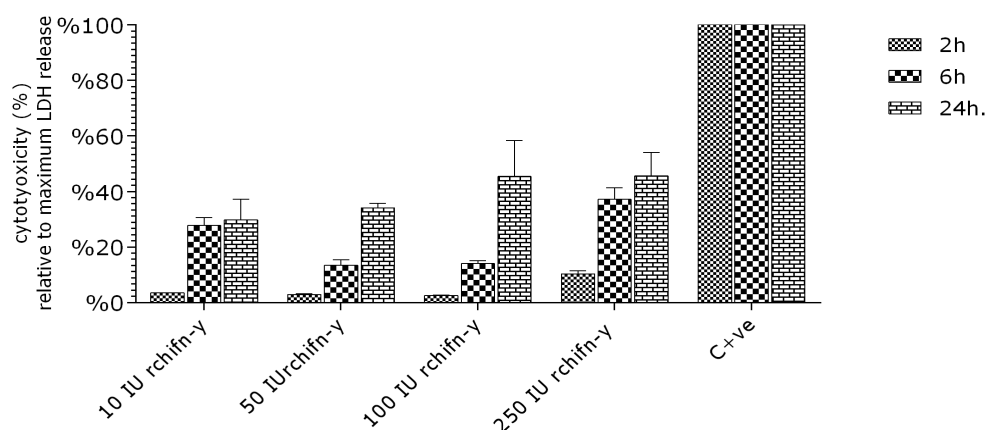


Figure 8.8: Assessment by LDH release of cytotoxicity of rchIFN-γ on HD11 cells. The cytotoxicity (%) was measured over 24h of treatment. Values shown as (mean $\pm$ SE) for three separate experiments with internal triplicate repeats. The doses were chosen upon the minimum cytotoxicity and maximum nitrite concentration productions indicated by Griess assays.

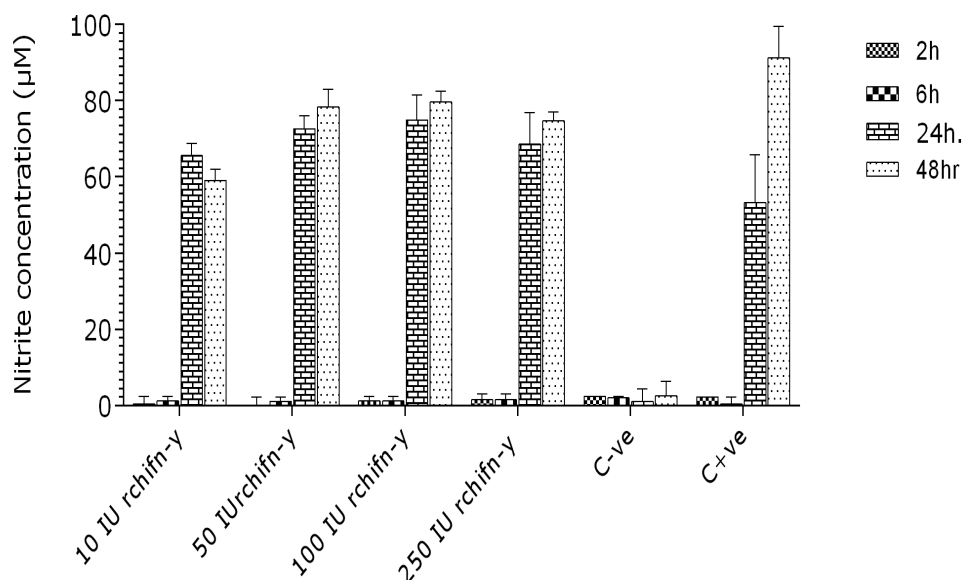


Figure 8.9: Optimization of recombinant chicken gamma interferon rchIFN-γ on inducing nitric oxide production by LPS-treated HD11 cells. The cells were incubated with different concentration of rchIFN-γ for 2h then the treated cells were challenged with 50 μ l of LPS at 100 ng/ml and compared to the controls. Positive controls of HD11 were treated with 50 μ l of LPS at 50 μ g/ml as a final concentration and negative controls wells of mock-infected were challenged with 50 μ l of PBS. Assays were done at 2, 6, 24 and 48h post-treatment. The values shown are the mean $\pm$ SE for three separate experiments with internal triplicate repeats.

8.6 Optimization of PMA for ROS production

The concentration of ROS stimulator phorbol-12-myristate-13-acetate (PMA) to stimulate HD11 and THP-1 cells was chosen according to its cytotoxic effects to the cells and ROS levels compared with control of non-treated cells were challenged with 100 μ l of LPS at 50 μ g/ml and negative controls wells of mock-infected (PBS). For ROS production, different concentrations of PMA to trigger THP-1 cells for 48h and HD11 cells for 24h were used (Figure 8.11 and 8.13). Following the incubation period at different time points, the supernatants were kept for cytotoxicity assays. The cells were washed and prepared for the reactive oxygen species production assays. However, the data showed that PMA was very safe with no any cytotoxic effects at any used concentrations, 100 ng/ml of PMA was chosen and used during experiments in this study.

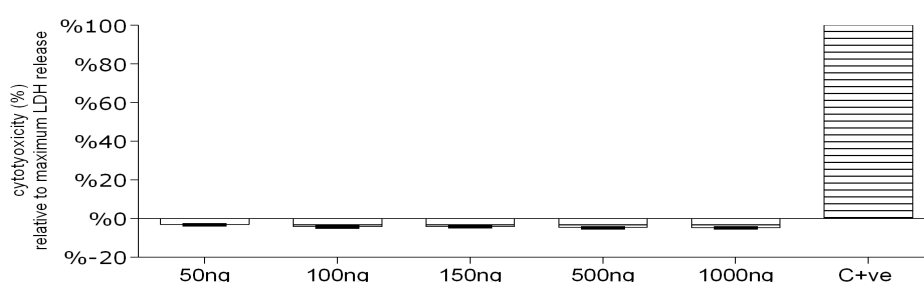


Figure 8.10: Assessment by LDH release of cytotoxicity of phorbol-12-myristate-13-acetate PMA on HD11 cells. Different concentrations of PMA were used to treat HD11 for 24h and LDH levels were determined after 2h post-treatment and compared with the positive control (100% of LDH released from cells lysed at 5×10^5 cells / ml were prepared through sequential freezing / thawing cycles). The values shown are the means $\pm$ SE for three separate experiments with internal triplicate repeats.

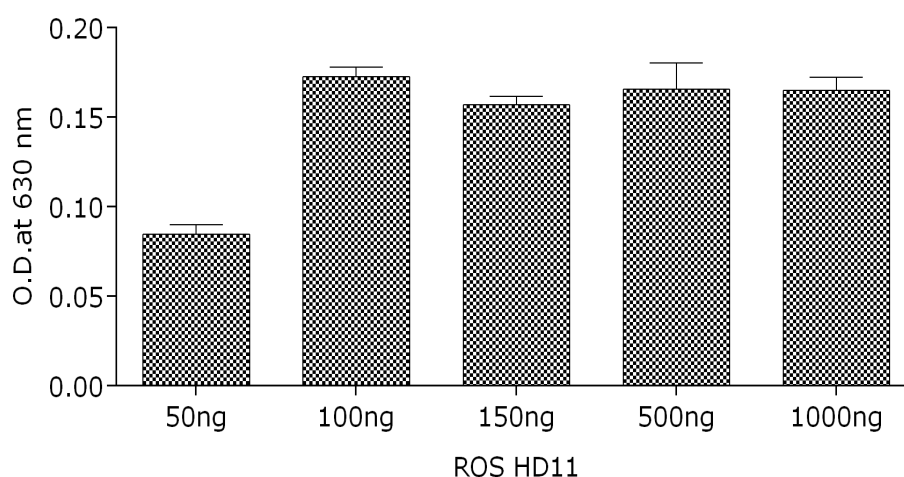


Figure 8.11: Optimization of phorbol-12-myristate-13-acetate PMA on inducing ROS production by LPS-treated HD11 cells. 5×10^5 cells / ml were incubated with different concentrations of PMA (ROS stimulator) for 24h then the treated cells were challenged with 100 μ l of LPS at 50 μ g/ml. ROS level is expressed as increases of the optical density at 630 nm (OD690 nm) above negative control. Assays were done at 2h after treatment. The values shown are the means $\pm$ SE for three separate experiments with internal triplicate repeats.

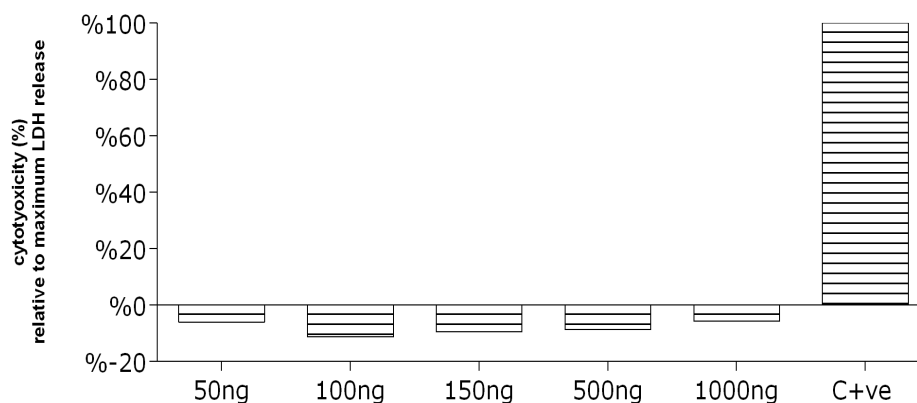


Figure 8.12: Assessment by LDH release of cytotoxicity of phorbol-12-myristate-13-acetate PMA on THP-1 cells. The cells were incubated with different concentrations of PMA for 48h and LDH were assayed at 2h post-treatment and compared with the positive control 100% of LDH released from cells lysed were prepared through sequential freezing / thawing cycles. The values shown are the means $\pm$ SE for three separate experiments with internal triplicate repeats

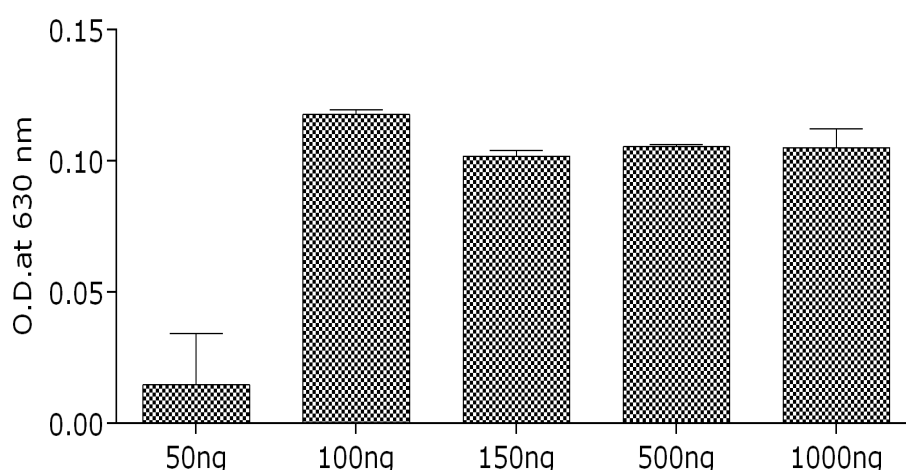


Figure 8.13: Optimization of phorbol-12-myristate-13-acetate PMA on inducing ROS production by LPS-treated THP-1 cells. 5×10^5 cells / ml were incubated with different concentrations of PMA (ROS stimulator) for 48h then the treated cells were challenged with 100 μ l of LPS at 50 μ g/ml. ROS level is expressed as increase of the optical density at 630 nm (OD_{630 nm}) above negative control. Assays were done at 2h post-treatment. The values shown are the means $\pm$ SE for three separate experiments with internal triplicate repeats.

8.7 An *in situ* calibration curve to measure phagosomal pH

To measure pH of phagosomes containing *M. avium* and Zymosan A in infected HD11 CF-Rho probes were used to label the five bacterial strains and Zymosan A. FLUOstar Optima was used to measure the CF/Rho emission ratio. The fluorescence units of each well were used to analysis data. The phagosomal pH was determined according to the standard calibration curve based on the CF/Rho ratio versus pH. An *in situ* calibration curve was made by incubating cells in different pH buffers, pH 4-8 in the presence of the ionophore nigericin to adjust the intracellular pH artificially to the values

defined by the incubation buffers. Figure 8.14 shows a representative calibration curve, independent calibration curves at each time point and for each experiment were established due to the differences in labelling intensities.

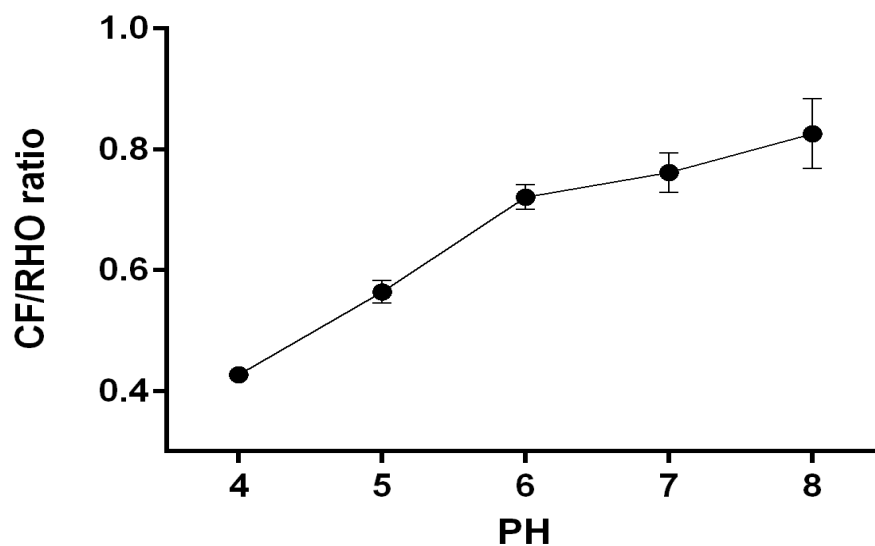


Figure 8.14: *In situ* calibration curve for the calculation of phagosomal pH from the CF/Rho emission ratio.

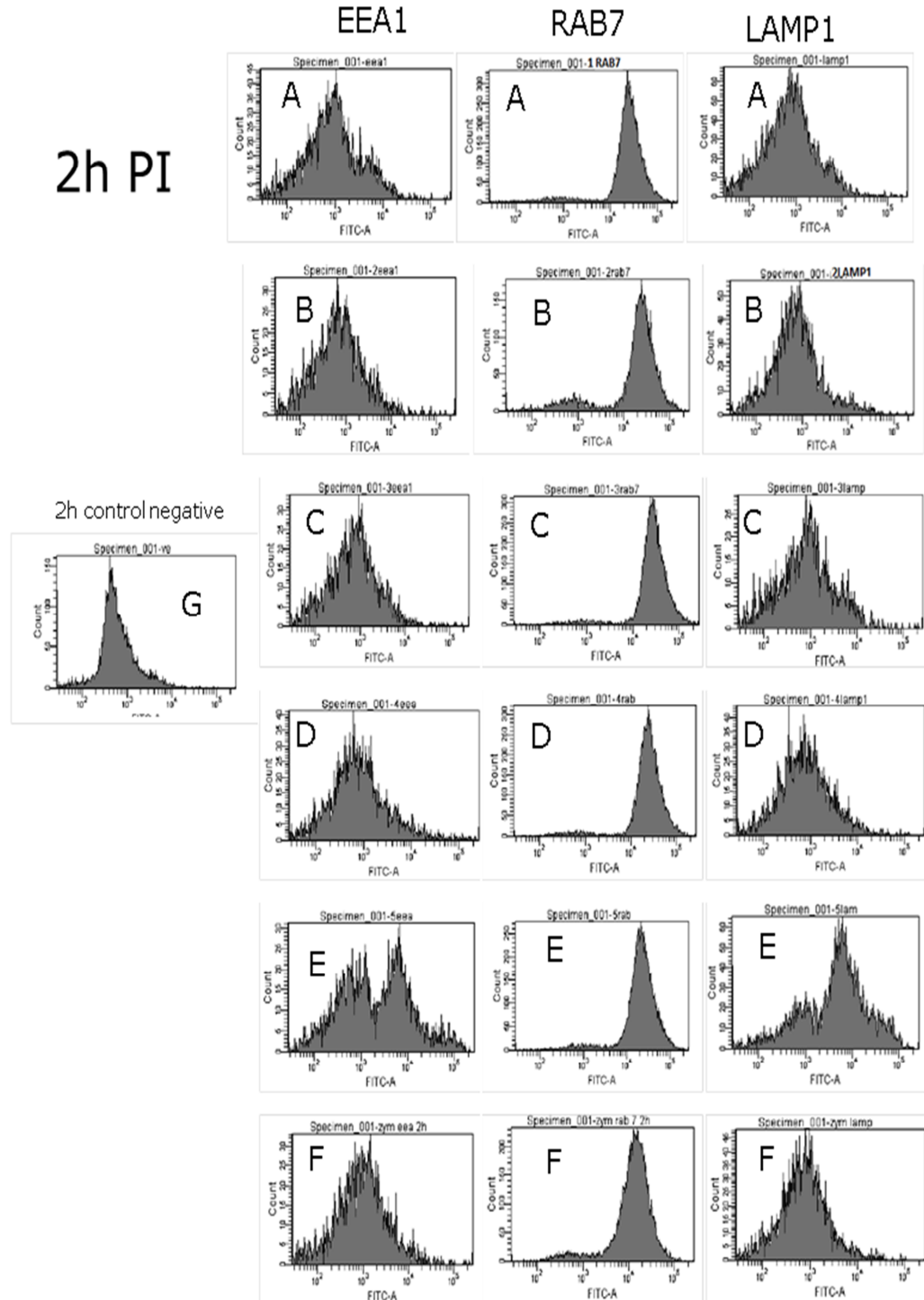


Figure 8.15: Represented histogram of association with phago-lysosomal markers of internalised *M. avium* and Zymosan A in HD11 cells at 2h post-infection. (A) *M. avium* Ma1 (NC08562.02), (B) Ma2 (NC08559-07), (C) Ma3 (61-3623-05), (D) Ma4 (61-4627.08), (E) Ma5 (61-3728.08), (F) Zymosan A and (G) control negative-non-infected cells. Rabbit anti-chicken polyclonal antibodies were used to recognize (EEA1, RAB7 and LAMP1 markers) % of positive populations were determined by flow cytometry.

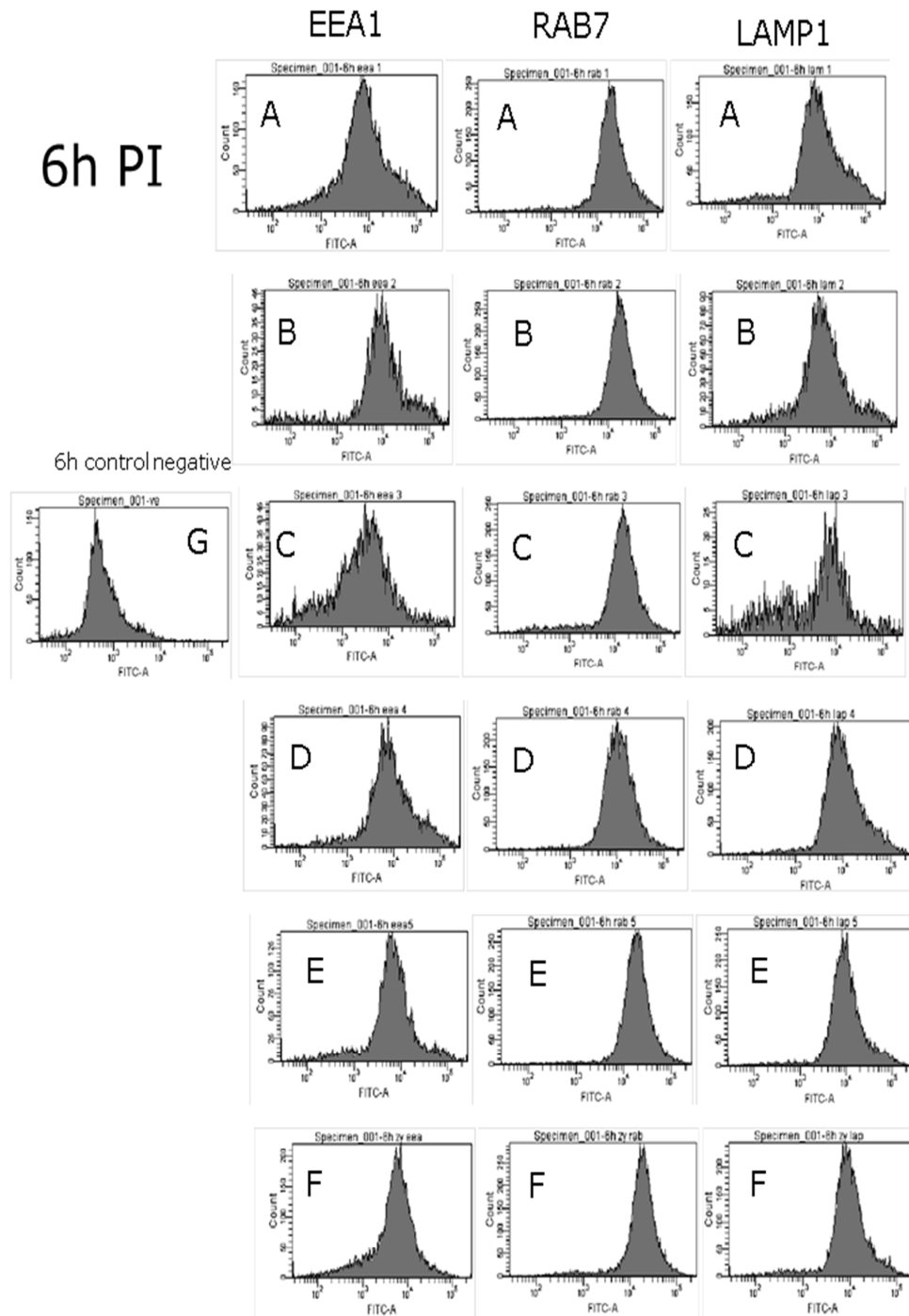


Figure 8.16: Represented histogram of association with phago-lysosomal markers of internalised *M. avium* and Zymosan A in HD11 cells at 6h post-infection. (A) *M. avium* Ma1 (NC08562.02), (B) Ma2 (NC08559-07), (C) Ma3 (61-3623-05), (D) Ma4 (61-4627.08), (E) Ma5 (61-3728.08), (F) Zymosan A and (G) control negative-non-infected cells. Rabbit anti-chicken polyclonal antibodies were used to recognize (EEA1, RAB7 and LAMP1 markers) % of positive populations were determined by flow cytometry.

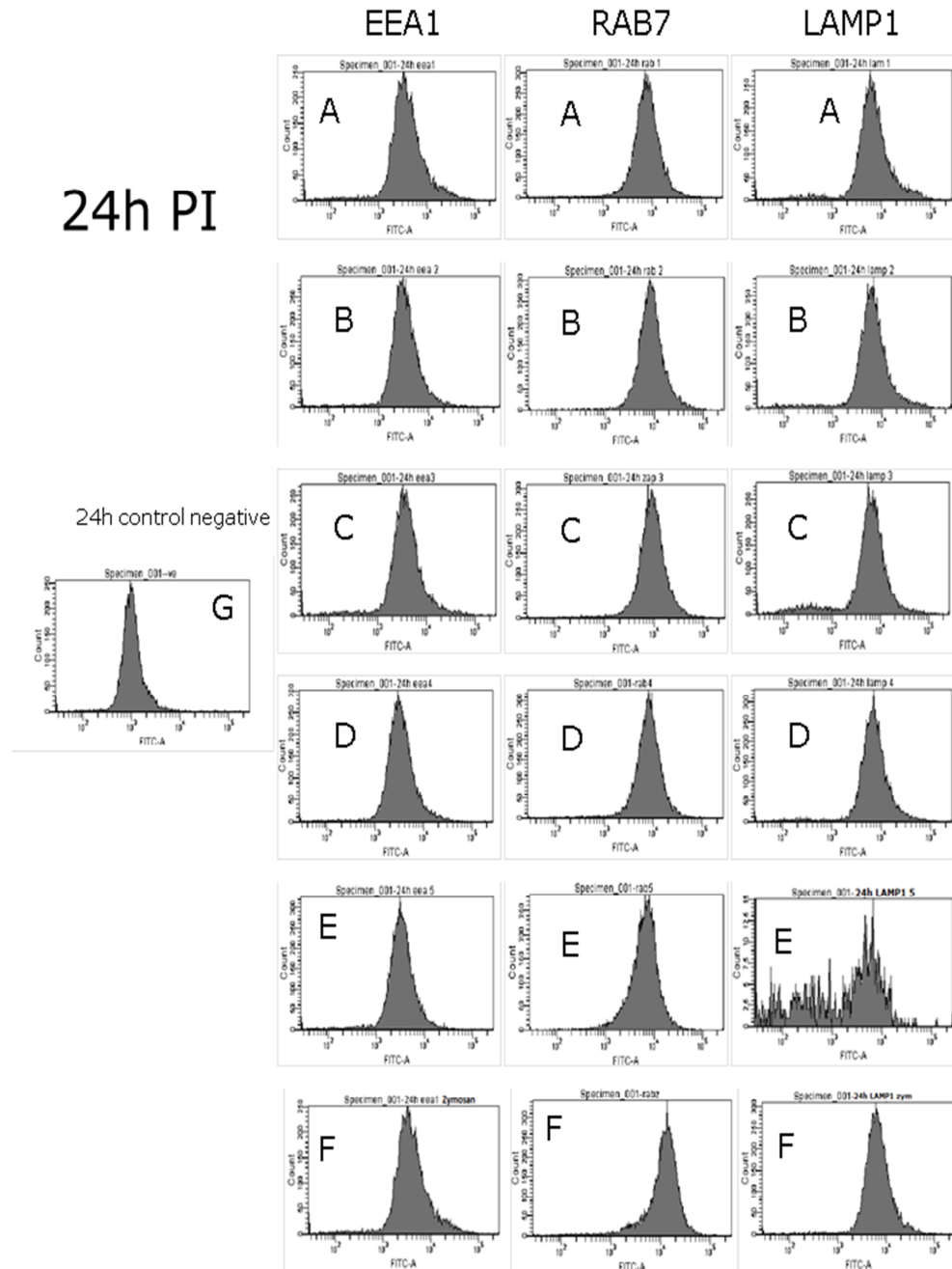


Figure 8.17: Represented histogram of association with phago-lysosomal markers of internalised *M. avium* and Zymosan A in HD11 cells at 24h post-infection. (A) *M. avium* Ma1 (NC08562.02), (B) Ma2 (NC08559-07), (C) Ma3 (61-3623-05), (D) Ma4 (61-4627.08), (E) Ma5 (61-3728.08), (F) Zymosan A and (G) control negative-non-infected cells. Rabbit anti-chicken polyclonal antibodies were used to recognize (EEA1, RAB7 and LAMP1 markers) % of positive populations were determined by flow cytometry.

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